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(54) Title: A POLYPEPTIDE FROM LUNG EXTRACT WHICH BINDS AMYLOID-β PEPTIDE

(57) Abstract

The present invention provides for a method for inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycati n end product on the surface of a cell which comprises contacting the cell with an agent capable of inhibiting interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an amount effective to inhibit interaction of the amyloid-β peptide with the receptor for advanced glycation end product on the surface of the cell. Another embodiment of this invention is a method for evaluating the ability of an agent to inhibit binding of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which includes: a) contacting the cell with the agent and amyloid-β peptide; b) determining the amount of amyloid-β peptide bound to the cell and c) comparing the amount of bound amyloid-β peptide determined in step b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit the binding of amyloid-β peptide to the receptor for advanced glycation end product on the surface of the cell.

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A POLYPEPTIDE
FROM LUNG EXTRACT WHICH BINDS AMYLOID-β PEPTIDE

5 This application claims the priority of U.S. Serial No. 08/592,070, filed January 26, 1996, the contents of which are hereby incorporated by reference into the present application.

10 The invention disclosed herein was made with Government support under USPHS Grants No. AG00690, AG00603, HL21006 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

15

Background of the Invention

Throughout this application, various publications are referenced by author and date. Full citations for these 20 publications may be found listed alphabetically at the end of the specification immediately preceding Sequence Listing and the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state 25 of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The pathologic hallmarks of Alzheimer's disease (AD) are intracellular and extracellular accumulations of proteins, 30 progression of which is closely correlated with eventual neuronal dysfunction and clinical dementia (for reviews see Goedert, 1993; Haass et al., 1994; Kosik, 1994; Trojanowski et al., 1994; Wischik, 1989). Amyloid-ß peptide (Aß), which is the principal component of extracellular deposits in AD, 35 both in senile/diffuse plaques and in cerebral vasculature, actively influences cellular functions as indicated by

several lines of evidence: Aß has been shown to promote
neurite outgrowth, generate reactive oxygen intermediates
(ROIs), induce cellular oxidant stress, lead to neuronal
cytotoxicity, and promote microglial activation (Behl et
5 al., 1994; Davis et al., 1992; Hensley, et al., 1994; Koh,
et al., 1990; Koo et al., 1993; Loo et al., 1993; Meda et
al., 1995; Pike et al., 1993; Yankner et al., 1990). For Aß
to induce these multiple cellular effects, cell surfaces may
contain a binding protein(s) which engages Aß.   In this
10 context, several cell-associated proteins, as well as
sulfated proteoglycans, can interact with Aß.   These
include: substance P receptor, the serpin-enzyme complex
(SEC) receptor, apolipoprotein E, apolipoprotein J
(clusterin), transthyretin, alpha-1 anti-chymotrypsin, ß-
15 amyloid precursor protein, and sulphonates/heparin sulfates
(Abraham et al., 1988; Fraser et al., 1992; Fraser et al.,
1993; Ghiso et al., 1993; Joslin et al., 1991; Kimura et
al., 1993; Kisilevsky et al., 1995; Strittmatter et al.,
1993a; Strittmatter et al., 1993b; Schwarzman et al., 1994;
20 Snow et al., 1994; Yankner et al., 1990).   Of these, the
substance P receptor and SEC receptor might function as
neuronal cell surface receptors for Aß, though direct
evidence for this is lacking (Fraser et al., 1993; Joslin et
al., 1991; Kimura et al., 1993; Yankner et al., 1990).   In
25 fact, the role of substance P receptors is controversial,
and it is not known whether Aß alone interacts with the
receptor, or if costimulators are also required (Calligaro
et al., 1993; Kimura et al., 1993; Mitsuhashi et al., 1991)
and the SEC receptor has yet to be fully characterized.
30 Amyloid-ß peptide (Aß) is central to the pathology of
Alzheimer's disease (AD), primarily because of its
neurotoxic effects which involve induction of cellular
oxidant stress.

Summary of the Invention

The present invention provides for a method for inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell

5 which comprises contacting the cell with an agent capable of inhibiting interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an amount effective to inhibit interaction of the amyloid-β peptide with the receptor for advanced glycation

10 end product on the surface of the cell. Another embodiment of this invention is a method for evaluating the ability of an agent to inhibit binding of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which includes: a) contacting the cell with the

15 agent and amyloid-β peptide; b) determining the amount of amyloid-β peptide bound to the cell and c) comparing the amount of bound amyloid-β peptide determined in step b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit the binding

20 of amyloid-β peptide to the receptor for advanced glycation end product on the surface of the cell.

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Brief Description of the Figures

Figures 1A, 1B, 1C, 1D, 1E and 1F. Colocalization of heme oxygenase type I (HO-1; A,D), p50 antigen (B, E) and RAGE
5 (C,F) in proximity to deposits of Aß in AD brain. Figures 1A, 1B, and 1C demonstrate increased expression of HO-1, p50 and RAGE antigens, respectively, in temporal lobe. The red color shows the antigen under study (HO-1, p50 or RAGE) and the black color depicts localization of Aß. Arrows in
10 Figure 1B depict nucelar localization of p50. Figures 1D-1F display the same antigens in cerebrovasculature from AD-derived brain. Marker bar: 50 μm (1A,1C,1D,1F); 30 μm (B); 125 μm (E). Figure 1G shows ELISA for RAGE antigen in homogenates of AD brain versus age-matched apparently normal
15 control.

Figures 2A, 2B, 2C-1, 2C-2, 2D-1, 2D-2, 2E and 2F. Aß-induction of cellular oxidant stress and binding involves association with a cell surface, trypsin-sensitive
20 polypeptide on endothelium (A,C,E) and cortical neurons (B,D,F). A,C,E. Endothelial cells were incubated with the indicated concentration of synthetic Aß (1-40) or ^{125}I-Aß, and induction of cellular oxidant stress was assessed by the TBARS assay (A) or radioligand binding studies were
25 performed (C-1,E). Where indicated, cultures were pre-treated with probucol or N-acetylcysteine (NAC), or briefly exposed to trypsin (Figure 2C-2: +, treated with trypsin; -, controls not treated with trypsin). In Figure 2E, cross-linking of ^{125}I-Aß (100 nM) to the cell surface was
30 performed with DSS. Lanes correspond to: (1) ^{125}I-Aß alone + endothelium (no DSS); (2) ^{125}I-Aß with excess unlabelled Aß (100-fold), DSS and endothelium; and (3) ^{125}I-Aß with DSS and endothelium; and, Figures 2B, 2D, 2F show the same experiments performed with primary cultures of rat cortical
35 neurons instead of endothelium. Note that in Figure 2F, samples are: (1) as in E, above; (2) ^{125}I-Aß with DSS and

cortical neurons; and, (3) ^{125}I-Aß with excess unlabelled Aß, DSS and cortical neurons.

Figures 3A, 3B and 3C. Purification of cellular Aß-binding polypeptide(s). Tissue extract was subjected to chromatography on heparin (A) and hydroxylapatite (B) columns. OD_{28c} of the fractions (solid line), the concentration of salt in the buffer (broken line), and fractions with peak ^{125}I-Aß binding activity (arrow/bar) are shown. In Figure 3C, fractions with peak ^{125}I-Aß binding activity from the hydroxylapatite column were subjected to SDS-PAGE (10%; nonreduced), and stained with Coomassie blue or sliced into 2 mm pieces, eluted, and assayed for specific ^{125}I-Aß binding activity.

Figures 4A, 4B, 4C, 4D, and 4E. Binding of ^{125}I-Aß (1-40) and related peptides to RAGE: dose-dependence (A-B), effect of RAGE blockade (C) and competition (D-E). A-B. Binding of ^{125}I-Aß to purified RAGE immobilized in microtiter wells was studied: 4A, the indicated concentration of RAGE was incubated for adsorption to wells (^{125}I-Aß, 100 nM); and 4B, the indicated concentration of ^{125}I-Aß was added to wells pre-incubated with RAGE (5 µg/well). Where indicated, wells with adsorbed RAGE were preincubated with trypsin as described in the text (Figure 4A). 4C, Binding of ^{125}I-Aß (100 nM) to RAGE adsorbed to microtiter wells was studied in the presence of the indicated concentration of sRAGE (2-, 20- or 50-fold molar excess), sVCAM-1 (20x), anti-RAGE IgG (10 or 50 µg/ml) or nonimmune IgG (10 µg/ml). Figure 4D-E. Competitive binding studies. Plates were coated with RAGE (5 µg/well) as above. In Figure 4D, wells were incubated with ^{125}I-Aß (100 nM) in the presence of one of the following unlabelled competitors (in each case, an 100-fold molar excess of unlabelled peptide to ^{125}I-Aß was added): Arg-Gly-Asp-Ser (Seq. I.D. No. 1)(RGDS), scrambled Aß (25-35)', Aß (25-35), Aß (1-20), aggregated Aß (1-40), Aß

(1-40, fresh), and 0 denotes no addition. In Figure 4E, binding studies utilized ^{125}I-Aß (100 nM) and an 100-fold excess of unlabelled Aß-derived from AD brain vasculature or plaque core, or synthetic Aß (1-40). Data shown represent
5 percent maximal specific binding, and the mean±SEM of triplicate determinations is shown. Maximal specific binding (100%) is that observed with ^{125}I-Aß (1-40; 100 nM) alone minus ^{125}I-Aß + 100-fold molar excess unlabelled Aß (1-40; freshly prepared). Data were analyzed by ANOVA
10 Fisher's PLSD and the P values are * <0.01; ^ <0.02.

Figures 5A, 5B and 5C. Role of RAGE in binding of ^{125}I-Aß to mouse endothelial cells and cortical neurons: effect of sRAGE and anti-RAGE IgG. Figure 5A. Effect of sRAGE on
15 binding of ^{125}I-Aß to endothelium and cortical neurons. Figure 5B-C. Effect of anti-RAGE IgG, at the indicated concentration, on binding of ^{125}I-Aß to endothelium (B) or cortical neurons (C). In each case, cell cultures were incubated with ^{125}I-Aß (100 nM) alone or in the presence of
20 the indicated concentrations of sRAGE, anti-RAGE IgG or nonimmune IgG (NI; 20 μg/ml). The mean±SEM of quintuplicate determinations is shown. P values shown are # <0.01 and *<0.05

25 Figures 6A, 6B, 6C and 6D. Effect of RAGE blockade on Aß-induced cellular oxidant stress in endothelial cells (A,C) and PC12 cells (B,D). Figure 6A-B. Generation of TBARS. Endothelial (A) or PC12 (B) cells were incubated with Aß (1 μM) either alone or in the presence of the
30 indicated amounts of anti-RAGE IgG, nonimmune IgG (NI; 20 μg/ml) or sRAGE (100 μg/ml). P values shown are *<0.01 and # <0.05. Figure 6C-6D. Activation of NF-kB. The same cells were incubated with Aß alone or with other additions (as above), nuclear extracts were prepared and studied by
35 EMSA. For endothelial cells (C) and PC12 cells (D): Lane 1, ^{32}P-labelled NF-kB probe; 2, cells not exposed to Aß; 3,

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cells exposed to Aß (500 nM); 4-5, cells exposed to Aß (500 nM; 4) in the presence of anti-RAGE IgG or nonimmune IgG (10 µg/ml in each case; lane 5); 6, extracts of cells exposed to Aß (500 nM) in the presence of 100-fold excess unlabelled
5 NF-KB probe.

Figures 7A, 7B, 7C, 7D, 7E, 7F, 7G and 7H. Effect of RAGE blockade on Aß-induced cellular stress in PC12 cells. Cultures were exposed to the indicated concentrations of Aß
10 (25-35) alone, or in the presence of either sRAGE, anti-RAGE IgG, or nonimmune (NI) IgG. Figure 7A-D. The effect of RAGE blockade on MTT reduction is shown: A, MTT reduction at different concentrations of Aß in the presence/absence of sRAGE (20-fold molar excess over Aß); B, MTT reduction at
15 different concentrations of sRAGE (Aß , 1 µM); and, C-D, MTT reduction at different concentrations of Aß, anti-RAGE IgG or nonimmune IgG (10 µg/ml). P values shown are *<0.01 and #<0.05. In Figure 7D, the concentration of Aß is 1 µM. Figures 7E-H show micrographs depicting PC12 cells either
20 incubated in medium alone (E) or medium containing the following: Aß (1 µM; F); Aß (1 µM) + sRAGE (50 µg/ml; G); or Aß (1 µM) + anti-RAGE IgG (20 µg/ml; H). Addition of nonimmune IgG in place of anti-RAGE IgG had no effect on Aß-induced morphologic changes in PC12 cells. Marker bar:
25 100 µm.

Figures 8A-8B. RAGE expression by microglial cells. A-B. Colocalization of CD68. Figure 8A shows mouse anti-human CD68 IgG (4 µg/ml) and RAGE. Figure 8B shows rabbit
30 anti-human RAGE IgG (30 µg/ml) in microglia approximate to a senile plaque from AD brain (adjacent sections). Immunostaining was performed using the indicated primary antibodies.

35 Figures 9A-9B. BV-2 cells bind ^{125}I-Aß in a RAGE-dependent manner. Figure 9A. Dose dependence of binding. Figure 9B.

Inhibition of ^{125}I-Aß binding by blockade of RAGE using anti-RAGE F(ab')$_2$ or sRAGE, at the indicated concentrations, but not by nonimmune F(ab')$_2$. BV-2 cells (5x10^4/well) in minimal essential medium with bovine serum albumin (1%) were
5   incubated with either ^{125}I-Aß alone (1.3x10^4 cpm/ng; prepared by the Iodogen method) or in the presence of one of the indicated additions at 4˚C for 2 hrs. Wells were washed with Hanks balanced salt solution seven times, and cell-associated radioactivity was eluted with Nonidet P-40
10 (1%). Specific binding (total minus nonspecific) is shown and equilibrium binding data was analyzed as described using Enzfitter (Schmitdt, et al. 1992). Synthetic peptide was from Quality Controlled Biochemicals and AD-derived Aß was prepared (Roher, et al. 1993).
15

Figures 10A, 10B and 10C. Modulation of BV-2 migration by Aß: role of RAGE. Figure 10A. Soluble Aß (1-40 and 25-35) induces chemotaxis of BV-2 cells in dose-dependent manner: effect of anti-RAGE F(ab')$_2$ or nonimmune F(ab')$_2$ on Aß(1-40
20 and 25-35)-induced chemotaxis. Figure 10B. Haptotoxis: effect of Aß or albumin adsorbed to the upper surface of chemotaxis chamber membranes on migration of BV-2 cells to fMLP added to the lower chamber. Figure 10C. Anti-RAGE F(ab')$_2$ blocks haptotactic response to Aß(1-40) adsorbed to
25 chemotaxis chamber membranes. BV-2 cells were preincubated with either anti-RAGE F(ab')$_2$ or nonimmune F(ab')$_2$ for 1 hr at 4˚C, cells were washed with Hanks balanced salt solution, and then added to the upper compartment of chemotoxis chambers in which the upper surface of membranes were coated
30 with Aß(1-40). BV-2 cells (5x10^3/well) were added to the upper compartment of microchemotaxis chambers for 4 hrs at 37˚C, and then cells reaching the lower surface of the chemotaxis chamber membrane were visualized with Wright's stain and counted. Data are reported as cells per
35 high-power field (HPF) based on counting nine fields/well and each experiment was performed in triplicated. Where

indicated, the upper surface of the membranes was coated
with Aß(1-40) by floating in an Aß solution (50 nM) for 3
hrs at 37°C, followed by washing and drying prior to use
(Schmidt, et al. 1993).

5

Figures 11A, 11B and 11C.   Aß-induced TNFa production and
NF-kB activation in BV-2 cells is prevented by blocking
access to RAGE.   Figure 11A.   Aß-induction of TNFα
transcripts by PCR.   BV-2 cells (10^6) were incubated with
10 Aß(25-35; 500 nM) for 4 hr at 37°C, and RNA was harvested.
RT-PCR was performed using primers for murine TNFα
(Clontech) and the housekeeping gene mouse ß-actin
(Clontech) was stimultaneously amplified.   Ethidium
bromide-stained gels are shown.   The positive control (the
15 respective cDNA) and migration of standard is shown.   Where
indicated, BV-2 cells were pre-incubated with anti-RAGE
F(ab')$_2$ or nonimmune (NI) F(ab')$_2$ (10 µg/ml in each case) for
1 hr at 37°C.   Figure 11B.   Aß(25-35; 1 µM) induction of
TNFα antigen in BV-2 cells.   Cultures were incubated for 18
20 hrs at 37°C with Aß, and cell-free supernatants were assayed
for TNFα antigen (Biosource).   Where indicated, BV-2 cells
were exposed to anti-RAGE F(ab')$_2$ or nonimmune F(ab')$_2$ as
above.   Figure 11C.   Aß(25-35)-induction of NF-kB activation
in BV-2 cells.   Lanes represent: (1) free probe (FP); (2)
25 BV-2 cells with medium alone; (3) BV-2 cells + Aß; (4-5)
BV-2 cells + Aß with anti-RAGE F(ab')$_2$ (2 µg/ml [4] and 10
µg/ml [5]); (6) BV-2 cells + Aß and nonimmune F(ab')$_2$ (10
µg/ml); and, (7) BV-2 cells + Aß studied in the presence of
an 100-fold excess of unlabelled NF-kB probe.   Cultures (10^7
30 cells) were exposed to Aß (1 µM) for 4 hrs at 37°C, nuclear
extracts were prepared, and gel retardation assay was
performed using a double-stranded DNA oligonucleotide for
the consensus NF-kB site.   Where indicated, BV-2 cells were
pre-incubated with anti-RAGE F(ab')$_2$ or nonimmune F(ab')$_2$ (10
35 µg/ml) for 1 hr at 37°C and then washed twice prior to
exposure to Aß.

<u>Detailed Description of the Invention</u>

The present invention provides for a method for inhibiting
interaction of an amyloid-β peptide with a receptor for
5   advanced glycation end product on the surface of a cell
which comprises contacting the cell with an agent capable of
inhibiting interaction of the amyloid-β peptide with the
receptor for advanced glycation end product, the agent being
present in an amount effective to inhibit interaction of the
10 amyloid-β peptide with the receptor for advanced glycation
end product on the surface of the cell.

In the present invention the cell may be a neuronal cell, an
endothelial cell, a glial cell, a microglial cell, a smooth
15 muscle cell, a somatic cell, a bone marrow cell, a liver
cell, an intestinal cell, a germ cell, a myocyte, a
mononuclear phagocyte, an endothelial cell, a tumor cell, or
a stem cell. The agent may be a peptide, a peptidomimetic,
a nucleic acid or a small molecule. The peptide may be at
20 least a portion of the sequence -Asp-Ala-Glu-Phe-Arg-His-
Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-
Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-
Gly-Gly-Val-Val- (Seq. I.D. No. 2). The peptide may be at
least a portion of the sequence -Gly-Ser-Asn-Lys-Gly-Ala-
25 Ile-Ile-Gly-Leu-Met-(Seq. I.D. No. 3).

The agent may be a soluble receptor for advanced glycation
end product. The agent may be a soluble extracellular
portion of a receptor for advanced glycation end product, an
30 antibody or portion thereof, wherein the antibody is capable
of specifically binding to the receptor for advanced
glycation endproduct. The antibody may be a monoclonal
antibody or a polyclonal antibody. A portion of the
antibody may be a Fab or a complementarity determining
35 region or a variable region. The agent may be capable of
specifically binding to the amyloid-β peptide. The agent

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may bind to the amyloid-β peptide at the site where the receptor for advanced glycation end product interacts.

One embodiment of this invention is a method for inhibiting
5   degeneration of a neuronal cell which includes contacting the cell with an agent capable of inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of the neuronal cell.

10 Another embodiment of this invention is a method for inhibiting formation of an amyloid-β peptide fibril on a cell which includes contacting the cell with an agent capable of inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the
15 surface of the cell.

Another embodiment of this invention is a method for inhibiting extracellular assembly of an amyloid-β peptide into a fibril which includes contacting the amyloid-β
20 peptide with an agent capable of inhibiting interaction of an amyloid-β peptide with another amyloid-β peptide.

Another embodiment of this invention is a method for inhibiting aggregation of amyloid-β peptide on the surface
25 of a cell which includes contacting the amyloid-β peptide with an agent capable of inhibiting interaction of the amyloid-β peptide with a receptor for advanced glycation end product.

30 Another embodiment of this invention is a method for inhibiting aggregation of amyloid-β peptide on the surface of a cell which includes contacting the receptor for advanced glycation end product with an agent capable of inhibiting interaction of the amyloid-β peptide with the
35 receptor for advanced glycation end product.

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Another embodiment of this invention is a method for
inhibiting infiltration of a microglial cell into senile
plaques which includes contacting the microglial cell with
an agent capable of inhibiting interaction of an amyloid-β
5 peptide with a receptor for advanced glycation end product
on the surface of the microglial cell.

Another embodiment of this invention is a method for
inhibiting activation of a microglial cell by an amyloid-β
10 peptide which includes contacting the microglial cell with
an agent capable of inhibiting interaction of the amyloid-β
peptide with a receptor for advanced glycation end product
on the surface of the microglial cell. The inhibition of
activation may include decreased production of cytokines by
15 the microglial cell. The interaction may be binding of the
amyloid-β peptide to the receptor for advanced glycation end
product on the surface of the cell.

One embodiment of this invention is a method for treating a
20 subject with a condition associated with interaction of an
amyloid-β peptide with a receptor for advanced glycation end
product on a cell, which comprises administering to the
subject an agent capable of inhibiting the interaction of
the amyloid-β peptide with the receptor for advanced
25 glycation end product, the agent being present in an amount
effective to inhibit the amyloid-β peptide interaction with
the receptor for advanced glycation end product on the cell
thereby treating the subject. The condition in this
embodiment may be diabetes, Alzheimer's Disease, senility,
30 renal failure, hyperlipidemic atherosclerosis, neuronal
cytotoxicity, Down's syndrome, dementia associated with head
trauma, amyotrophic lateral sclerosis, multiple sclerosis or
neuronal degeneration. The subject may be a mammal or a
human. The administration in this embodiment may be
35 intralesional, intraperitoneal, intramuscular or intravenous
injection; infusion; liposome-mediated delivery; topical,

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nasal, oral, anal, ocular or otic delivery.

In this embodiment, the condition may be associated with
degeneration of a neuronal cell in the subject, with
5 formation of an amyloid-β peptide fibril, with aggregation
of amyloid-β peptide, with infiltration of a microglial cell
into a senile plaque, or with activation of a microglial
cell by an amyloid-β peptide, wherein the activation
comprises production of cytokines by the microglial cell.
10

Another embodiment of this invention is a method for
evaluating the ability of an agent to inhibit binding of an
amyloid-β peptide with a receptor for advanced glycation end
product on the surface of a cell which includes: a)
15 contacting the cell with the agent and amyloid-β peptide; b)
determining the amount of amyloid-β peptide bound to the
cell and c) comparing the amount of bound amyloid-β peptide
determined in step b) with the amount determined in the
absence of the agent, thus evaluating the ability of the
20 agent to inhibit the binding of amyloid-β peptide to the
receptor for advanced glycation end product on the surface
of the cell.

In this embodiment, the cell may be contacted with the agent
25 and the amyloid-β peptide simultaneously or the cell may be
contacted with the amyloid-β peptide and the agent. The
agent may be capable of specifically binding to the amyloid-
β peptide. The agent may bind to amyloid-β peptide at the
site where the receptor for advanced glycation end product
30 interacts. The agent may be a soluble extracellular portion
of a receptor for advanced glycation end product. The agent
may be bound to a solid support. The agent may be expressed
on the surface of a cell.

35 Another embodiment of this invention is a method for
inhibiting activation of an NF-κB gene in a cell which

comprises contacting the cell with an agent which is capable
of inhibiting interaction of amyloid-β peptide with a
receptor for advanced glycation endproduct on the cell, thus
inhibiting activation of NF-κB in the cell.

5

The present invention provides for a pharmaceutical
composition which comprises an agent capable of inhibiting
interaction of amyloid-β peptide with a receptor for
advanced glycation endproduct and a pharmaceutically
10 acceptable carrier. The carrier may be a diluent, an
aerosol, a topical carrier, an aquous solution, a nonaqueous
solution or a solid carrier.

As used herein, the term "oxidant stress" encompasses the
15 perturbation of the ability of a cell to ameliorate the
toxic effects of oxidants. Oxidants may include hydrogen
peroxide or oxygen radicals that are capable of reacting
with bases in the cell including DNA. A cell under "oxidant
stress" may undergo biochemical, metabolic, physiological
20 and/or chemica modifications to counter the introduction of
such oxidants. Such modifications may include lipid
peroxidation, NF-kB activation, heme oxygenase type I
induction and DNA mutagenesis. Also, antioxidants such as
glutathione are capable of lowering the effects of oxidants.
25

As used herein, the term "neurotoxicity" encompasses the
negative metabolic, biochemical and physiological effects on
a neuronal cell which may result in a debilitation of the
neuronal celluar functions. Such functions may include
30 memory, learning, perception, neuronal electrophysiology
(ie. action potentials, polarizations and synapses), synapse
formation, both chemical and electrical, channel functions,
neurotransmitter release and detection and neuromotor
functions. Neurotoxicity may include neuronal cytotoxicity.
35

As used herein, the term "neuronal degeneration" encompasses

a decline in normal functioning of a neuronal cell. Such a
decline may include a decline in memory, learning,
perception, neuronal electrophysiology (ie. action
potentials, polarizations and synapses), synapse formation,
5 both chemical and electrical, channel functions,
neurotransmitter release and detection and neuromotor
functions. In the present invention, the subject may be a
mammal or a human subject. The administration may be
intralesional, intraperitoneal, intramuscular or intravenous
10 injection; infusion; liposome-mediated delivery; gene
bombardment; topical, nasal, oral, anal, ocular or otic
delivery.

In the practice of any of the methods of the invention or
15 preparation of any of the pharmaceutical compositions an
"therapeutically effective amount" is an amount which is
capable of inhibiting the binding of an amyloid-β peptide
with a receptor for advanced glycation endproduct.
Accordingly, the effective amount will vary with the subject
20 being treated, as well as the condition to be treated. For
the purposes of this invention, the methods of
administration are to include, but are not limited to,
administration cutaneously, subcutaneously, intravenously,
parenterally, orally, topically, or by aerosol.
25
As used herein, the term "suitable pharmaceutically
acceptable carrier" encompasses any of the standard
pharmaceutically accepted carriers, such as phosphate
buffered saline solution, water, emulsions such as an
30 oil/water emulsion or a triglyceride emulsion, various types
of wetting agents, tablets, coated tablets and capsules. An
example of an acceptable triglyceride emulsion useful in
intravenous and intraperitoneal administration of the
compounds is the triglyceride emulsion commercially known as
35 Intralipid®.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color
5 additives or other ingredients.

This invention also provides for pharmaceutical compositions including therapeutically effective amounts of protein compositions and compounds capable of inhibiting the binding
10 of an amyloid-β peptide with a receptor for advanced glycation endproduct in the subject of the invention together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers useful in treatment of neuronal degradation due to aging, a
15 learning disability, or a neurological disorder. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent
20 absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking
25 substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the compound, complexation with metal ions, or incorporation of the compound into or onto particulate preparations of polymeric compounds such as polylactic acid,
30 polglycolic acid, hydrogels, etc, or onto liposomes, micro emulsions, micelles, unilamellar or multi lamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the
35 compound or composition. The choice of compositions will

depend on the physical and chemical properties of the compound capable of alleviating the symptoms of the cognitive disorder of memory or the learning disability in the subject.

5

Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or

10 poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors

15 or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

Portions of the compound of the invention may be "labeled"

20 by association with a detectable marker substance (e.g., radiolabeled with ^{125}I or biotinylated) to provide reagents useful in detection and quantification of compound or its receptor bearing cells or its derivatives in solid tissue and fluid samples such as blood, cerebral spinal fluid or

25 urine.

When administered, compounds are often cleared rapidly from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent

30 injections of relatively large doses of bioactive compounds may by required to sustain therapeutic efficacy. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl

35 cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone

or polyproline are known to exhibit substantially longer
half-lives in blood following intravenous injection than do
the corresponding unmodified compounds (Abuchowski et al.,
1981; Newmark et al., 1982; and Katre et al., 1987). Such
5 modifications may also increase the compound's solubility in
aqueous solution, eliminate aggregation, enhance the
physical and chemical stability of the compound, and greatly
reduce the immunogenicity and reactivity of the compound.
As a result, the desired *in vivo* biological activity may be
10 achieved by the administration of such polymer-compound
adducts less frequently or in lower doses than with the
unmodified compound.

Attachment of polyethylene glycol (PEG) to compounds is
15 particularly useful because PEG has very low toxicity in
mammals (Carpenter et al., 1971). For example, a PEG adduct
of adenosine deaminase was approved in the United States for
use in humans for the treatment of severe combined
immunodeficiency syndrome. A second advantage afforded by
20 the conjugation of PEG is that of effectively reducing the
immunogenicity and antigenicity of heterologous compounds.
For example, a PEG adduct of a human protein might be useful
for the treatment of disease in other mammalian species
without the risk of triggering a severe immune response.
25 The compound of the present invention capable of alleviating
symptoms of a cognitive disorder of memory or learning may
be delivered in a microencapsulation device so as to reduce
or prevent an host immune response against the compound or
against cells which may produce the compound. The compound
30 of the present invention may also be delivered
microencapsulated in a membrane, such as a liposome.

Polymers such as PEG may be conveniently attached to one or
more reactive amino acid residues in a protein such as the
35 alpha-amino group of the amino terminal amino acid, the

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epsilon amino groups of lysine side chains, the sulfhydryl
groups of cysteine side chains, the carboxyl groups of
aspartyl and glutamyl side chains, the alpha-carboxyl group
of the carboxy-terminal amino acid, tyrosine side chains, or
5 to activated derivatives of glycosyl chains attached to
certain asparagine, serine or threonine residues.

Numerous activated forms of PEG suitable for direct reaction
with proteins have been described. Useful PEG reagents for
10 reaction with protein amino groups include active esters of
carboxylic acid or carbonate derivatives, particularly those
in which the leaving groups are N-hydroxysuccinimide, p-
nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-
sulfonate.   PEG derivatives containing maleimido or
15 haloacetyl groups are useful reagents for the modification
of protein free sulfhydryl groups. Likewise, PEG reagents
containing amino hydrazine or hydrazide groups are useful
for reaction with aldehydes generated by periodate oxidation
of carbohydrate groups in proteins.
20

The pathologic hallmarks of Alzheimer's disease (AD) are
intracellular and extracellular deposition of filamentous
proteins which closely correlates with eventual neuronal
dysfunction and clinical dementia (for reviews see Goedert,
25 1993; Haass et al., 1994; Kosik, 1994; Trojanowski et al.,
1994; Wischik, 1989). Amyloid-ß peptide (Aß) is the
principal component of extracellular deposits in AD, both in
senile/diffuse plaques and in cerebral vasculature. Aß has
been shown to promote neurite outgrowth, generate reactive
30 oxygen intermediates (ROIs), induce cellular oxidant stress,
lead to neuronal cytotoxicity, and promote microglial
activation (Behl et al., 1994; Davis et al., 1992; Hensley,
et al., 1994; Koh, et al., 1990; Koo et al., 1993; Loo et
al., 1993; Meda et al., 1995; Pike et al., 1993; Yankner et
35 al., 1990).   For Aß to induce these multiple cellular
effects, it is likely that plasma membranes present a

binding protein(s) which engages Aß.   In this context,
several cell-associated proteins, as well as sulfated
proteoglycans, can interact with Aß.   These include:
substance P receptor, the serpin-enzyme complex (SEC)
5 receptor, apolipoprotein E, apolipoprotein J (clusterin),
transthyretin, alpha-1 anti-chymotrypsin, ß-amyloid
precursor protein, and sulphonates/heparan sulfates (Abraham
et al., 1988; Fraser et al., 1992; Fraser et al., 1993;
Ghiso et al., 1993; Joslin et al., 1991; Kimura et al.,
10 1993; Kisilevsky et al., 1995; Strittmatter et al., 1993a;
Strittmatter et al., 1993b; Schwarzman et al., 1994; Snow et
al., 1994; Yankner et al., 1990). Of these, the substance
P receptor and SEC receptor might function as neuronal cell
surface receptors for Aß, though direct evidence for this is
15 lacking (Fraser et al., 1993; Joslin et al., 1991; Kimura et
al., 1993; Yankner et al., 1990).   In fact, the role of
substance P receptors is controversial, and it is not known
whether Aß alone interacts with the receptor, or if
costimulators are required (Calligaro et al., 1993; Kimura
20 et al., 1993; Mitsuhashi et al., 1991) and the SEC receptor
has yet to be fully characterized.

This invention is illustrated in the Experimental Details
section which follows. These sections are set forth to aid
25 in an understanding of the invention but are not intended
to, and should not be construed to, limit in any way the
invention as set forth in the claims which follow
thereafter.

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EXPERIMENTAL DETAILS

Example 1: A Receptor Mediating Amyloid-ß Peptide-induced Oxidant Stress and Neurotoxicity

Amyloid-ß peptide (Aß) is central to the pathology of Alzheimer's disease (AD), primarily because of its neurotoxic effects which involve induction of cellular oxidant stress. A cell-associated ≈50 kDa polypeptide which binds Aß peptide (1-40) was purified to homogeneity and was shown to be identical to RAGE, an immunoglobulin superfamily member which also serves as a receptor for both amphoterin, a group I DNA binding protein involved in neuritic outgrowth, and advanced glycation endproducts (AGEs). Recombinant RAGE bound ^{125}I-Aß (1-40) in a dose-dependent manner; binding was blocked by excess Aß purified from AD brain and by unlabelled synthetic Aß (1-40 and 25-35) but not by Aß (1-20). Binding of ^{125}I-Aß to cultured cortical neurons was prevented by anti-RAGE IgG or excess soluble RAGE. In addition, blocking access to RAGE prevented Aß-induced cellular oxidant stress and cytotoxicity. Increased expression of RAGE in neurons proximate to senile plaques in AD brain, which also display evidence of oxidant stress and cytotoxicity, further suggests the relevance of RAGE-Aß interaction to cellular dysfunction and ultimate neurodegeneration, and is consistent with the concept that this interaction might be critical in the pathogenesis of AD.

Introduction

The possibility exists that another/other cellular binding site(s) might be present which could tether Aß to the cell surface. The importance of identifying such receptors is evident from the probability that Aß closely associated with the cell surface might trigger reactions generating potent

oxidizing stimuli capable of inducing cytotoxicity. If this hypothesis is correct, blocking access of Aß to the cell surface binding site(s) might considerably retard progression of cellular dysfunction in AD.

5

This study reports the isolation and characterization of a ~50 kDa polypeptide which binds synthetic Aß peptide (1-40) and is identical to RAGE (the Receptor for Advanced Glycation Endproducts). RAGE, previously identified as a

10 central cellular receptor for Advanced Glycation Endproducts (AGEs; Schmidt et al. 1992; Neeper et al., 1992) also serves as a neuronal receptor for amphoterin, a molecule involved in neurite outgrowth (Rauvala et al., 1987). It is shown herein that RAGE mediates the interaction of Aß with

15 endothelial cells (ECs) and neuronal-like cells, as demonstrated by radioligand binding experiments, as well as by its ability to mediate cellular oxidant stress and neuronal cytotoxicity. Enhanced expression of RAGE in AD, both in affected neurons and the vasculature, is consistent

20 with the possibility that Aß-RAGE interaction may be relevant to the cytotoxicity central to this dementia.

Results

Relationship between oxidant stress markers and Aß in AD

25 brain. Previous studies have demonstrated an association between neuronal injury in AD and oxidant stress (Coyle et al., 1993; Hensley, 1994; Volicer et al., 1990) by expression of heme oxygenase type 1 (HO-1), nuclear localization of the p50 subunit of NF-kB, and expression of

30 malondialdehyde-lysine epitopes (Smith et al., 1994; Yan et al., 1994; Yan et al., 1995). Although extracellular Aß can induce oxidant stress in neurons *in vitro* (Behl et al., 1994; Hensley et al., 1994), this would probably be attenuated *in vivo* by the presence of abundant antioxidants.

35 Nevertheless, neurons proximate to senile plaques displayed HO-1, and elevated levels of the p50 subunit of the NF-kB

family (Fig. 1A-B, respectively). Increased expression of
p50 was especially prominent in a nuclear distribution (see
arrows in Fig 1B), suggestive of its activation. Deposition
of Aß also occurs in the cerebral vasculature (Glenner,
5 1979; Kawai et al., 1992; Luthert et al., 1991; Yamaguchi et
al., 1992); oxidant stress (increased HO-1 and p50) was also
evident in those vessels with accumulated Aß (Fig. 1D-E).
Expression of HO-1 and p50 was enhanced in both endothelial
cells and smooth muscle cells. In contrast, cerebral
10 vasculature from age-matched controls showed minimal
staining for HO-1 and p50. These observations suggested a
spatial association between Aß deposition and the expression
of markers of enhanced cellular oxidant stress in AD brain,
leading to the hypothesis that binding of Aß to specific
15 cellular structures might promote such perturbation of
cellular properties, in spite of the antioxidant milieu
prevalent in vivo.

Characterization of the interaction of Aß with ECs and
20 neuronal-like cells. Signs of oxidant stress in AD lead to
the interaction of Aß with vascular cells and with neurons,
and the consequences for perturbation of cellular functions.
Incubation of synthetic Aß (1-40) with ECs from murine
cerebral vasculature or rat cortical neurons cells resulted
25 in a dose-dependent increase in thiobarbituric acid-reactive
substances (TBARS), which was blocked by pre-treatment of
cultures with probucol or N-acetylcysteine (NAC) suggestive
of an oxidant-sensitive mechanism (Fig. 2A-B). These
results are consistent with what has been reported
30 previously with neurons exposed to Aß (Behl et al., 1994;
Harris et al., 1995; Mattson et al., 1995). To test the
possibility that Aß-induced cellular oxidant stress involved
interaction with a cell surface polypeptide, endothelial
cells or cortical neurons were briefly treated with trypsin,
35 the enzyme was neutralized and cells then exposed to Aß.
Trypsin treatment virtually completely blocked appearance of

TBARS (Fig. 2A-B), though it had no effect on detection of
TBARS when added after cells had been pre-incubated with Aß.
Similar experiments examining induction of HO-1 mRNA and
nuclear translocation of NF-kB showed cellular activation by
5 Aß, which was prevented by pretreatment of cultures with
trypsin. Studies with PC12 cells provided similar results.
These data strongly supported a role for a trypsin-sensitive
cell surface polypeptide in Aß-induction of cellular oxidant
stress.
10

Reasoning that such a cell surface structure was likely to
function, at least in part, as a binding site to tether Aß
to the cell, radioligand binding studies were performed.
When synthetic Aß peptide (residues 1-40) was radiolabelled,
15 specific and dose-dependent binding of ^{125}I-Aß to cultured
ECs was observed (Fig. 2C). Half-maximal binding occurred
at an ^{125}I-Aß concentration of 40.0±9.79 nM and, at
saturation, there were ≈1.7x10^4 molecules bound per cell.
Exposure of cultures to trypsin blocked binding of ^{125}I-Aß
20 (Fig. 2C, inset). Concentrations of ^{125}I-Aß which increased
occupancy of cell surface binding sites were comparable to
those inducing endothelial generation of TBARS (Fig. 2A).
Further supporting a central role for a cell surface binding
site is clear from the strong inhibitory effect of trypsin
25 on both ^{125}I-Aß binding and Aß-mediated oxidant stress.
Similarly, radioligand binding studies on cultured rat
cortical neurons displayed dose-dependent ^{125}I-Aß binding, K_d
55.2±14.6 nM, which was blocked by pre-treatment of cells
with trypsin (Fig. 2D).
30

To further identify the nature of cell surface
polypeptide(s) with which ^{125}I-Aß was interacting,
crosslinking studies were performed. ECs with surface-bound
^{125}I-Aß were exposed to disuccinimidyl suberate (DSS; 0.2 mM)
35 and extracts were prepared for SDS-PAGE and autoradiography
(Fig. 2E). A single major band, M_r ≈50-55 kDa was observed

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(Fig. 2E, lane 3), whose intensity was greatly attenuated upon addition of an 100-fold molar excess of unlabelled Aß (Fig. 2E, lane 2). Ruling out the possibility that this band was due to self-aggregation of the tracer, when ^{125}I-Aß

5 was incubated with DSS under the same conditions in the absence of ECs, no band was observed. Similar cross-linking studies on cortical neurons also showed that ^{125}I-Aß became associated with a polypeptide of M_r ~50-55 kDa (Fig. 2F, lane 2).

10

Before proceeding further with characterization of the ~55 kDa polypeptide, it was important to determine whether cell-associated ^{125}I-Aß was interacting with previously recognized Aß-binding proteins, such as apolipoproteins E

15 and J, heparin-like and heparan sulfate proteoglycans, substance P receptors, and transthyretin. Pre-treatment of ECs with heparin (10 μg/ml), which has been shown to release cell-associated apo E (Ji et al., 1994) or preincubation with heparanase (under conditions which blocked 80% of

20 ^{125}I-basic fibroblast growth factor binding to ECs, due to cell surface and matrix heparan sulfate proteoglycans; Moscatelli, 1992) did not affect ^{125}I-Aß binding to endothelium. Pilot studies in which substance P (added as an unlabelled competitor) or antibody to transthyretin was

25 present during the incubation of ^{125}I-Aß with ECs had no effect on binding. Apo J, with M_r ~80 kDa (Ghiso et al., 1993), was unlikely to be responsible for the ~55 kDa band observed on SDS-PAGE following crosslinking of ^{125}I-Aß to the cell surface. These studies suggested that the

30 cell-associated Aß binding polypeptide under study here was distinct from previously described species.

Isolation and characterization of Aß cell-associated binding proteins. In order to purify cellular Aß binding proteins,

35 a binding assay was developed employing extracts of cultured cells or tissues immobilized on microtiter wells and ^{125}I-Aß.

The first studies used ECs, as their binding of ^{125}I-Aß was
similar to that of neurons, and because they are easier to
culture and obtain in larger numbers. ECs were extracted
with octyl-ß-glucoside in the presence of protease
5 inhibitors, diluted in carbonate/bicarbonate buffer, and the
mixture was adsorbed to microtiter wells. Specific binding
of ^{125}I-Aß was similar to that observed on cultured ECs: it
was dose-dependent (K_d 32.9±10.2 nM) and completely prevented
by pre-treatment of the cell extract with trypsin. To
10 further scale up starting material for purification of
cell-associated Aß binding proteins, acetone-extract of
bovine lung was tested using a similar assay following
adsorption to microtiter wells and incubation with ^{125}I-Aß.
Again, similar specific, trypsin-sensitive, and
15 dose-dependent binding was observed. Although extract of
brain, especially from patients with AD, would have been a
more appropriate starting material for purification of Aß
binding polypeptides, studies showed that brain extract,
though in many respects comparable to lung extract, was not
20 a convenient starting material. Results of binding assays
with brain extract were not reproducible, probably due to
the high content of lipid, and human brain, especially
patient-derived tissue, was not available in sufficient
quantity.
25

The preparation of Aß binding protein(s) from lung was
performed by subjecting acetone extract of the tissue to
sequential chromatography on heparin and hydroxylapatite
columns. Following application of tissue extract, the
30 heparin column was washed with equilibration buffer and
eluted with ascending concentrations of NaCl (Fig. 3A).
^{125}I-Aß binding activity was observed in the eluate, as
indicated (maximal activity, 0.5 M NaCl), and fractions were
pooled, dialyzed and applied to hydroxylapatite. The column
35 was eluted with increasing concentrations of phosphate, and
fractions corresponding to 0.25 M displayed the highest

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binding activity for ^{125}I-Aß (Fig. 3B). This material was
concentrated and applied to preparative nonreduced SDS-PAGE
(10%), and two major bands were observed corresponding to
M_r's of 30-35 and 50-60 kDa (Fig. 3C). Another lane of the
5 same gel loaded with the same sample was sliced into 2 mm
pieces, and each piece subjected to gel elution; the eluate
was studied in the ^{125}I-Aß binding assay. Each of the two
major protein bands demonstrated the capacity to
specifically bind ^{125}I-Aß (Fig. 3C). In contrast, gel
10 elution of lanes in which molecular weight standards were
run in place of test samples revealed no binding of ^{125}I-Aß.

Using the same gel system as above, bands were cut-out and
subjected to N-terminal sequence analysis. The ≈50 kDa band
15 was sequenced first as it was most abundant. The sequence
revealed virtual identity with bovine RAGE (Table 1).

Table 1 - Amino acid sequences of the ≈50KdA GEL BAND AND
30-35 kDa gel band: Comparison to RAGE

RAGE NH₂-terminal	DQNITARIGKPLVLNCKGAPKKPPQQLEWK[a]
NH₂-terminal ≈50kDa gel	DQXITARIGKPLVLNXKGAPKKPPQQLEW(K)[b]
NH₂-terminal 30-35 kDa gel band	DQXITARIGKPLVLNXKGAP[c]

The following abbreviations were used: X, no residue
identifiable at that cycle; parenthesis, tentative
assignment. (Neeper et al. 1992). [a]Seq. I.D. No. 8; [b] Seq.
25 I.D. No. 9; [c]Seq. I. D. No. 10)

Based on protein structure deduced from the bovine RAGE cDNA
and immunoblotting results (Neeper et al., 1992; Brett et
al., 1993), the ≈50 kDa band would most likely correspond to
30 full-length RAGE. The 30-35 kDa band also demonstrated the
same N-terminal sequence as RAGE, and most likely
represented the extracellular domain of RAGE which has been
observed previously to undergo proteolysis during
purification (Schmidt et al., 1992). Consistent with these
35 data, both 30-35 and ≈50 kDa polypeptides were reactive with

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anti-RAGE IgG prepared against recombinant human RAGE. A
minor, more slowly migrating peak of Aß binding activity,
corresponding to M_r ~66 kDa (Fig. 3C), gave two N-terminal
sequences, that for RAGE and albumin.

5

Binding of ^{125}I-Aß to RAGE. The interaction of RAGE with Aß
was further characterized by radioligand binding studies,
using purified recombinant human RAGE (a form consisting of
the extracellular domain termed soluble or sRAGE) expressed
10 in a baculovirus system (Hori et al., 1995). Following
adsorption of RAGE to microtiter wells, specific binding of
^{125}I-Aß (binding observed with^{125} I-Aß alone minus that
observed with ^{125}I-Aß and excess unlabelled Aß) was dependent
on the concentration of RAGE incubated with the wells (Fig.
15 4A). No specific binding was observed when RAGE was
replaced by albumin. Exposure of RAGE-coated wells to
trypsin (Fig. 4A) completely blocked subsequent binding of
^{125}I-Aß, as expected from our previous observation that RAGE
is inactivated by trypsin (Schmidt et al., 1992). When the
20 concentration of RAGE adsorbed to wells was held constant,
and varying amounts of ^{125}I-Aß were added, binding was
dose-dependent with half-maximal occupancy of binding sites
at 56.8 ± 13.9 nM (Fig. 4B). This was similar to the range of
Aß concentrations resulting in binding to and oxidant stress
25 in endothelial cells, PC12 cells and cortical neurons.
Addition of either anti-RAGE IgG or soluble RAGE
simultaneous with ^{125}I-Aß resulted in dose-dependent
inhibition of binding (Fig. 4C), whereas nonimmune (NI)
rabbit IgG or another immunoglobulin superfamily molecule,
30 vascular cell adhesion molecule-1 (VCAM-1; Osborn, 1989) was
without effect. These data indicate that RAGE binds
synthetic ^{125}I-Aß peptide (1-40).

Competition studies were performed to assess the region of
35 Aß (1-40) interacting with RAGE and to evaluate the effect
of Aß purified from AD brain (Fig. 4D-E). In the presence

of freshly-prepared unlabelled Aß (1-40), binding of ^{125}I-Aß
to RAGE was blocked (Fig. 4D). Competition was also
observed if Aß (1-40) was allowed to incubate for 3 days at
37°C, conditions which promote aggregation of the peptide
5 (Hensley et al., 1994; Lorenzo et al., 1994). A truncated
form of Aß (1-40) consisting of residues 25-35 also
inhibited binding of ^{125}I-Aß to RAGE. In accord with the
specificity of Aß-RAGE interaction, addition of excess
unlabelled Aß (1-20), scrambled Aß (25-35), or an irrelevant
10 peptide such as Arg-Gly-Asp-Ser (Seq. I.D. No. 1) had no
effect on ^{125}I-Aß binding to RAGE (Fig. 4D). Furthermore,
human Aß (1-40/1-42), chromatographically-purified from
neuritic plaques or vascular amyloid from brains of patients
with AD (Roher et al., 1993a; Roher et al., 1993b),
15 inhibited the binding of ^{125}I-Aß (Fig. 4E).

As a first step in understanding whether RAGE would mediate
the binding of Aß to cells, radioligand binding studies were
performed with cultured mouse brain endothelial cells (Fig.
20 5A,5B) and rat cortical neurons (Fig. 5A,5C). In each case,
binding of ^{125}I-Aß was blocked by addition of excess soluble
RAGE or anti-RAGE IgG in a dose-dependent manner. These
data indicated that RAGE functioned as a binding site for
^{125}I-Aß on cellular surfaces.
25

RAGE and Aß-induced cellular stress. A series of
experiments suggested that Aß interaction with RAGE mediates
cellular perturbations resulting in oxidant stress and
cytotoxicity. Endothelial cells exposed to Aß generated
30 TBARS (Fig. 2A and 6A); this was prevented by blocking
access to RAGE using either anti-RAGE IgG or excess soluble
receptor (Fig. 6A). Another index of oxidant stress
triggered by Aß, activation of NF-kB in ECs, was also
suppressed by blocking RAGE (Fig. 6C, compare lanes 3 and
35 4). Similar experiments on PC12 cells with Aß showed
generation of TBARS (Fig. 6B) and activation of NF-kB (Fig.

6D, compare lanes 3 and 4), also prevented by blocking
interaction of Aß with the receptor.

Because Aß is well-known for its toxicity to a range of
5 cultured neurons and neuronal-related cells (Behl et al.,
1994; Harris et al., 1995; Hensley et al., 1994; Koh et al.,
1990; Loo et al., 1993; Lorenzo et al., 1994; Pike et al.,
1993; Yankner et al., 1990), it was important to determine
if Aß-RAGE interaction mediated such effects of the peptide.
10 Incubation of PC12 cells with Aß (25-35) resulted in
dose-dependent   cytotoxic/stress-associated   changes,
including   perturbation   of   cellular   reduction   of
3-(4,5-dimethylthiazol-2-yl)-2,5,   diphenyl   tetrazolium
bromide (MTT; Fig. 7A). This biochemical index of cellular
15 stress was accompanied by morphologic changes, including
retraction of cellular processes (Fig. 7, compare panels
E-F). Addition of sRAGE (Fig. 7A-B, G) or anti-RAGE IgG
(Fig. 7C-D, H) prevented Aß-induced cellular stress, based
on each of these criteria. Similar results were observed
20 when PC12 cells were incubated with Aß(1-40).

RAGE expression in AD. The above data suggested that RAGE,
if present and/or up-regulated in cells likely to be
critical in the pathogenesis of AD, might participate in
25 mediating toxic effects when associated with Aß and related
peptides. ELISA of homogenates of AD brain demonstrated
~2.5-fold increase in patients compared with age-matched
controls (Fig. 1G).   Immunocytochemical studies showed
increased expression of RAGE (Fig. 1C) in neurons displaying
30 evidence of oxidant stress (Fig. 1A-B) proximate to deposits
of Aß.   In the vasculature, RAGE expression was also
enhanced in the vessel wall (Fig. 1F), in cellular elements
showing increased HO-1 and p50 (Fig. 1D-E) in the vicinity
of Aß deposition. Closer examination of vessel walls showed
35 RAGE to be enhanced in both endothelium and smooth muscle
cells.   In contrast, RAGE expression was minimal in

age-matched cerebral vasculature from apparently normal controls. Thus, RAGE expression appears to be significantly upregulated in neuronal elements around or participating in the neuritic plaques as well as in the vascular cells of the
5 affected AD brain.

Discussion

RAGE is a member of the immunoglobulin superfamily of cell
10 surface molecules originally identified by its capacity to bind advanced glycation endproducts (AGEs; Schmidt et al., 1992; Neeper et al., 1993). These irreversibly nonenzymatically glycated adducts, which form on free amino groups, accumulate in tissues during normal aging, and their
15 deposition is accelerated in diabetes, renal failure, and settings in which macromolecular turnover is delayed (Baynes, 1991; Ruderman et al., 1992). As AGEs have been localized to the vasculature, especially in diabetes and experimental hyperlipidemic atherosclerosis (Nakamura et
20 al., 1993; Palinski et al., 1995), the first characterization of RAGE in these studies emphasized its role as a cell surface receptor for AGEs on endothelial cells and mononuclear phagocytes. AGE-RAGE interaction mediates perturbation of central properties of each of these
25 cell types for vascular homeostasis: vascular permeability is increased, endothelial expression of VCAM-1 is induced, monocyte migration is stimulated and production of proinflammatory cytokines, such as tumor necrosis factor-alpha occurs (Schmidt et al., 1993; Schmidt et al.,
30 1995; Wautier et al., 1995;).

In addition to the vasculature, expression of RAGE is especially prominent in the central nervous system (Brett et al., 1993; Hori et al., 1995). During the embryonic and
35 early postnatal period in rat brain, RAGE is highly expressed by cortical neurons in hippocampus (P5) and

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cerebellum (P17). These observations strongly suggested that AGEs were almost certainly not the primary ligands of RAGE, and led to studies to seek another ligand which could engage RAGE and affect cellular function, distinct from
5 those consequent on AGE-RAGE interaction. This ligand proved to be amphoterin, a high mobility group I DNA binding protein which is highly expressed in developing neurons and has been shown to mediate neurite outgrowth when present extracellularly (Rauvala et al., 1987). Amphoterin binds to
10 RAGE specifically and with high affinity (K_d ~9 nM; Hori et al., 1995); radioligand binding studies with ^{125}I-amphoterin showed RAGE to be the receptor mediating its binding to cortical neurons, as well as subsequent induction of neurite outgrowth. Furthermore, neurons containing RAGE in
15 developing rat cerebral cortex also expressed, or were contiguous to other cells which expressed amphoterin, suggesting that amphoterin-RAGE interaction was occurring under physiologic conditions (Hori et al., 1995). Although detailed neuroanatomical studies of RAGE expression have not
20 yet been done, the receptor clearly displays more limited expression in the adult central nervous system compared with the early perinatal period. In adult animals, RAGE is found in a population of cortical neurons yet to be characterized and in spinal motor neurons (Brett et al., 1993). In
25 Alzheimer's disease, expression of RAGE within cortical neurons increases and is more widespread, especially in those neurons proximate to deposits of Aß and those cells bearing neurofibrillary tangles. This suggested that there was yet another ligand for RAGE under pathologic conditions,
30 such as in AD brain.

As findings concerning RAGE expression in AD were emerging, independent investigation into how Aß induced cellular oxidant stress was ongoing. Aß itself has been shown to
35 generate ROIs (Hensley et al., 1994) and Aß interaction with PC12 cells additionally triggers intracellular formation of

oxidants (Behl et al., 1994), such as hydrogen peroxide, resulting in perturbation of a range of cellular properties. Expression of markers associated with cellular oxidant stress in AD brain has been demonstrated, including HO-1,

5 activation of NF-kB and the presence of malondialdehyde-lysine epitopes (Smith et al., 1994; Yan et al., 1994; Yan et al., 1995). As antioxidant mechanisms abound in the extracellular space, it seemed that Aß engagement of a cell surface polypeptide would tether Aß to

10 the membrane where it could exert its oxidant effects in proximity to cellular structures. Consistent with this concept, binding of Aß to cortical neurons parallelled the induction of cellular oxidant stress, and both were blocked by pre-treatment of cultures with trypsin. Two lines of

15 investigation, one to understand the role of RAGE in AD brain and the other to identify cellular binding site(s) for Aß that mediate induction of cellular oxidant stress, converged when the N-terminal sequence of the cellular Aß binding protein proved to be identical to RAGE. Experiments

20 with recombinant RAGE and blocking antibody to the receptor further confirmed that RAGE specifically bound Aß and mediated, in large part, Aß binding to cortical neurons, as well as Aß induction of oxidant stress and neurotoxicity.

25 The interaction of Aß with RAGE was dose-dependent and specific, and appeared to involve the region comprising residues 25-35 of the peptide. As this portion of Aß has been shown to participate in Aß-mediated cytotoxicity and self-aggregation (Loo et al., 1993; Hensley et al., 1994;

30 Mattson et al., 1995; Shearman et al., 1994), it is possible that RAGE might act as a magnet to localize forming amyloid fibrils to the cell surface. Such a role for RAGE would emphasize its capacity for promoting the cytotoxicity of Aß, but may reflect an initially protective mechanism, intended

35 to mediate clearance of Aß monomers or small aggregates, gone awry in a sea of large Aß aggregates in AD brain.

Addition of excess extracellular domain of RAGE (sRAGE)
completely blocked ^{125}I-Aß binding to both ECs and cortical
neurons. When present in sufficient molar excess to Aß,
sRAGE also completely prevented Aß-induced neuronal
5 toxicity; at higher levels of Aß, e.g. 10 μM, sufficient
concentrations of soluble receptor could not be achieved for
complete neuroprotection. Anti-RAGE IgG prevented ^{125}I-Aß
binding to cortical neurons by only ~70%, whereas under the
same conditions it completely blocked ^{125}I-Aß binding to ECs.
10 Several cell surface polypeptides/proteoglycans have been
previously postulated to interact with Aß, including SEC,
substance P receptors and heparan sulfate proteoglycans,
although their role in the pathobiology of AD is not well
established. The inability to completely block specific
15 binding of ^{125}I-Aß to cortical neurons suggests that these or
other binding sites may also be involved, in addition to
RAGE. It is important to note that sRAGE, once bound to Aß,
appears to block cellular interactions of amyloid-ß peptide,
whether the latter is present as monomer or under conditions
20 favoring aggregation. This could reflect the size of sRAGE,
an μ30 kDa polypeptide compared with Aß (~4 kDa), but it
also might be due to binding in the vicinity of Aß (25-35).
It is possible that sRAGE can diminish cellular interactions
and toxicity of Aß deposits by preempting neuronal
25 interactions (with cell surface RAGE and, potentially, other
cell surface binding sites) and possibly by limiting
assembly of Aß into aggregates/fibrils. As blocking fibril
formation may delay pathologic changes in AD, and as
sRAGE-Aß monomers or smaller aggregates might turn over more
30 rapidly, a detoxification mechanism is suggested.

Aß is an oxidizing agent, as are AGEs, and such ligands
could exert their cellular effects simply as the result of
being tethered to RAGE on the cell surface. However, recent
35 studies have shown that stimulation of this receptor by
other ligands which do not themselves generate ROIs, induces

intracellular generation of oxidants in target cells, suggesting that recruitment of signal transduction mechanisms may occur following ligand-RAGE interaction. Further support for an intimate relationship between RAGE
5 and ROIs is the presence of three NF-kB sites in the RAGE promoter whose activity can be triggered by oxidants. These data suggest a possible feedback loop whereby binding of Aß to RAGE induces oxidant stress which further up-regulates RAGE, thereby augmenting further Aß-RAGE interaction. The
10 presence of nonenzymatically glycated aggregates of Aß, which has also been shown in AD (Vitek et al., 1994), might additionally facilitate the interaction of extracellular Aß with RAGE.

15 Although these studies represent only a first step in establishing a link between expression of RAGE and pathophysiologic changes in AD, they suggest a novel paradigm in which engagement of RAGE has quite different outcomes depending on the nature of the ligand and the
20 microenvironment. During the prenatal and early postnatal period, amphoterin-RAGE interaction promotes neurite outgrowth and supports normal development. In this context, small quantities of soluble Aß, which exists in normal individuals, might promote neurite outgrowth in association
25 with RAGE. In the vicinity of neuritic plaques, where dystrophic neurites are present, Aß-RAGE could mediate a repair mechanism or stimulate de novo neurite outgrowth. However, engagement of RAGE on cortical neurons in the presence of higher levels of Aß in AD, and, by analogy, RAGE
30 binding of AGEs in diabetic vasculature, could result in sustained cellular oxidant stress with deleterious consequences for cellular function and organic homeostasis.

Experimental Procedures
35

Cell culture, binding assays, and cross-linking studies.

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Murine microvascular endothelial cells (ECs) were used in this studies (Gumkowski et al., 1987). Cultured rat cortical neurons, obtained from neonatal animals and cultured as described previously (Yavin et al., 1974) were
5 used by day 17. At this time, cultures consisted of >90% neurons based on neurofilament staining. PC12 cells were obtained from ATCC (Rockville MD).

Aß (1-40), obtained from Quality Controlled Biochemicals
10 (Hopkinton MA), was dissolved in distilled water (1 mg/ml) and radioiodinated by the Iodobead procedure (Pierce, Rockford IL) as described by the manufacturer. The reaction mixture was desalted by gel filtration, and ^{125}I-Aß was >90% precipitable in trichloroacetic acid (20%) and migrated as
15 a single band with M_r ~4 kDa on tris/tricine gel (16%) electrophoresis. Radioligand binding studies were performed immediately after labelling using endothelial cells and cortical neurons as above. Because of detachment of cortical neurons from the growth substrate during pilot
20 binding experiments, cultures were briefly exposed to paraformaldehyde (2% for 15 min) prior to the binding studies. Cultures were washed with phosphate-buffered saline (calcium-magnesium-free), followed by addition of binding buffer (minimal essential medium containing bovine
25 serum albumin-fatty acid free [1%]). Tracer, ^{125}I-Aß, was added either alone, in the presence of an 100-fold molar excess of unlabelled Aß or with one of the reagents listed below, and cultures were incubated (37°C for 2 hrs for neurons and 4°C for 3.5 hrs for ECs) to allow binding. This
30 incubation time was sufficient for binding to reach an apparent maximum at the lowest tracer concentrations. Unbound ligand was removed by four rapid washes (a total of 6 secs/well); during washing, only a negligible amount of cell-bound ligand dissociated. Elution buffer (NaCl, 0.1 M,
35 containing Nonidet P-40, 1%) was then added to wells for 5 min at 37°C, and the liquid aspirated and counted. Specific

binding was defined as total binding (counts observed in wells incubated with ^{125}I-Aß alone) minus nonspecific binding (counts observed in wells incubated with ^{125}I-Aß in the presence of an 100-fold excess of unlabelled Aß).
5 Equilibrium binding data were analyzed according to the equation of Klotz and Hunston (Klotz et al., 1971) using nonlinear least-squares analysis. Where indicated, one of the following was added to binding mixtures: substance P (Sigma), rabbit anti-bovine RAGE IgG (Schmidt et al., 1992),
10 nonimmune rabbit and human IgG (Sigma), recombinant soluble human RAGE, recombinant soluble murine vascular cell adhesion molecule-1 (VCAM-1), antibody to transthyretin (Sigma), and unlabelled Aß-related peptides (1-40 and 25-35). Note that anti-bovine RAGE IgG has been previously
15 shown to interact with rat, human and bovine RAGE (Brett et al., 1993; Hori et al., 1995). In certain experiments, cells were pretreated with heparin (10 μg/ml for 30 min), heparanase (2.5 U/ml for 30 min; Sigma; Moscatelli, 1992), or TPCK-treated trypsin (0.05 units/ml for 30 min and serum
20 was added to neutralize trypsin prior to the binding assay with ^{125}I-Aß; Sigma). None of these procedures resulted in cell detachment or loss of viability, based on trypan blue uptake or detachment from the growth substrate. Cross-linking experiments were performed on ECs and neurons
25 (for these studies neurons were not exposed to paraformaldehyde) following binding of ^{125}I-Aß to the cell surface by adding disuccinimidyl suberate (DSS; 0.2 mM; Pierce) for 30 min at 25°C. Cultures were then washed twice and dissolved in lysis buffer (NaCl, 0.1 M; NP-40, 1%, PMSF,
30 2 mM, and leupeptin, 10 μg/ml) at 4°C for 1 hr. Cellular debris was removed by centrifugation (10,000 rpm for 10 min) and the supernatant was prepared for nonreduced SDS-PAGE (10%).

35 Endothelial cell protein extracts were prepared as described (Schmidt et al., 1992) from ≈10^{8} cells using

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octyl-ß-glucoside in the presence of protease inhibitors.
Binding of ^{125}I-Aß to proteins in the extract was studied by
a previously described method (Schmidt et al., 1992) using
an incubation time of 3 hrs at 37°C. Other additions were
5   as indicated above; note that Aß (1-20), scrambled Aß
peptide (25-35) was from Quality Controlled Biochemicals,
Arg-Gly-Asp-Ser (Seq. I.D. No. 1) was from Sigma, and Aß
purified from AD brain plaque and vasculature was prepared
as described (Roher et al., 1993a,b). For certain
10 experiments, after adsorption of sRAGE to the wells and
blocking with albumin, trypsin was added (0.5 units/ml for
1 hr; the initial amount of sRAGE added was 120 μg/ml), and
neutralization of residual enzyme was as described above.

15 <u>Purification of Aß binding proteins and characterization of
Aß interaction with RAGE</u>. Acetone extracts of bovine lung
and brain (Sigma) were diluted in carbonate/bicarbonate
buffer, adsorbed to microtiter wells (as above for
endothelial cell extract), and adsorbed proteins studied for
20 their ability to bind ^{125}I-Aß by the procedure described
above. For purification of Aß binding proteins, lung
extract (100 g) was dissolved in tris-buffered saline
containing octyl-ß-glucoside (1%) and PMSF (2 mM) overnight
at 4°C with constant agitation. The mixture was centrifuged
25 (10,000 rpm for 30 min), applied to Heparin HyperD® (bed
volume 40 ml; Biosepra), and the column eluted with
increasing concentrations of NaCl. Fractions were studied
in the microtiter assay for ^{125}I-Aß binding activity and
those with maximal activity were pooled, dialyzed, and then
30 loaded on hydroxylapatite-ultragel (30 ml bed volume;
Biosepra). The column was eluted with increasing
concentrations of phosphate, fractions with maximal ^{125}I-Aß
binding activity were identified, pooled and applied to
preparative nonreduced SDS-PAGE (10%). Gels were stained
35 with Coomassie blue, and identical lanes of the gel were
either cut into 2 mm slices, protein was eluted and studied

for Aß binding activity as above, or slices of the gel were subjected to N-terminal sequence analysis (Hori et al., 1995).

5 A DNA fragment coding for human sRAGE was obtained from a lung library by PCR using human recombinant soluble RAGE was prepared from the human RAGE cDNA (primers: 5'GATGGCAGCCGGAACAGCAGTT3' (Seq. I.D. No. 4) and 5'CTCAAGTTCCCAGCCCTGATCCTCC3') (Seq. I.D. No. 5) and cloned

10 into the pBacPAK8 vector (Clontech) for expression of recombinant protein as described (Hori et al., 1995). Recombinant sRAGE was purified to homogeneity from Sf9 media by chromatography on SP Sepharose fast flow and Superdex 200PG chromatography as described (Hori et al., 1995)

15 (Pharmacia) equilibrated with sodium phosphate (20 mM using a baculovirus expression system as described previously for amphoterin (Hori et al., 1995). Antibody was prepared to human recombinant sRAGE by immunizing rabbits according to standard procedures (Vaitukaitis, 1981), and purified IgG

20 was characterized as monospecific by immunoblotting and ELISA. A DNA fragment coding for the first three immunoglobulin-like domains of VCAM-1 was obtained from a lung cDNA library using PCR (primers: 5'GAAATGCCTGTGAAGATGGTCG3' (Seq. I.D. No. 6) and

25 5'CTCATTGAACACTAATTCCACTTC3') (Seq. I.D. No. 7), expressed and purified as above for sRAGE except that Q Sepharose (Pharmacia) was used (20 mM, pH 8.0).

Aß-RAGE induced cellular oxidant stress and cytotoxicity.
30 Evidence of Aß-induced cellular perturbation was studied by evaluating generation of TBARS (Dennery et al., 1990) and reduction of MTT (Promega, Madison WI). In addition, cultures were examined using an inverted Olympus PX-10 microscope. Electrophoretic mobility shift assays (EMSA)

35 were performed to assess nuclear translocation of NF-kB (Yan et al., 1995). Extracts of nuclear protein were derived

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from cultured cells, as indicated, and reacted with a consensus ^{32}P-labelled human NF-kB probe.

Immunohistologic studies were performed on paraffin sections
5 of formalin-fixed temporal lobe from AD and age-matched controls (Yan et al., 1995). Sections were first stained with either rabbit anti-human RAGE IgG (20 µg/ml), rabbit anti-human heme oxygenase type I (StressGen, Vancouver Canada), or rabbit anti-human p50 IgG (StressGen), and sites
10 of binding of primary antibody were visualized using the avidin/biotin alkaline phosphatase method. Double staining of these sections for Aß was performed using murine monoclonal anti-human Aß IgG (5 µg/ml), followed by peroxidase-conjugated goat anti-mouse IgG. Nonimmune rabbit
15 or mouse IgG were used as controls.

Example 2 : A Receptor Mediating Activation of Microglia by
Amyloid-β peptide.
Infiltration of ß-amyloid plaques by reactive microglia in
20 Alzheimer's disease Itagaki et al., 1989; Dickson et al., 1993; and McGeer et al., 1993) as well as ß-amyloid induced activation of microglia in vitro (Davis et al., 1992; Klegeris et al., 1994; Meda et al., 1995; and Araujo and Cotman, 1992),. suggests that ß-amyloid-microglial
25 interaction involves specific pathways.   RAGE, an immunoglobulin superfamily receptor previously shown to engage advanced glycation endproducts (Schmidt et al., 1992; and Neeper et al., 1992), serves as a specific binding site for ß-amyloid (1-40 and 25-35), and that its expression is
30 enhanced in reactive microglia in Alzheimer's brain.   RAGE has a central role in ß-amyloid-induced microglial activation: blockade of RAGE prevents binding of ^{125}I-ß-amyloid(1-40) to the cell surface; induction of microglial migration by soluble ß-amyloid and arrest of
35 microglia by immobilized ß-amyloid are mediated by RAGE; and stimulation of microglial expression of tumor necrosis

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factor-a and activation of NF-kB by ß-amyloid is due to
binding to RAGE.   These data suggest that blockade of
ß-amyloid-RAGE-dependent microglial activation might prevent
triggering of chronic inflammatory cascades in Alzheimer's
5 brain.

The association of activated microglia with amyloid plaques
in the brain of patients with Alzheimer's disease (AD) is
consistent with an ongoing immune/inflammatory response, as
10 evidenced by microglial expression of the class II major
histocompatibility antigen HLA-DR, and cytokines, such as
Interleukins 1 and 6 (Dickson et al., 1993; and McGeer et
al., 1993).   That changes in microglial activation might be
a   direct   consequence   of   ß-amyloid   (Aß)-microglial
15 interaction is supported by the observation that soluble
Aß(1-40 and 25-35) promotes microglial chemotaxis, as well
as TNF, Interleukin 1ß, and basic fibroblast growth factor
expression and nitrite production (Davis et al., 1992;
Klegeris et al., 1994; Meda et al., 1995; and Araujo and
20 Cotman, 1992).   Recently it has been found that RAGE,
Receptor for Advanced Glycation Endproducts (Schmidt et al.,
1992; and Neeper et al., 1992), binds Aß(1-40 and 25-35) in
a specific, dose-dependent, and saturable manner (K_d μ55 nM);
and Aß-RAGE interaction mediates, at least in part, neuronal
25 cytotoxicity and oxidant stress due to Aß (Yan et al.,
1996).   As RAGE is expressed by mononuclear phagocytes
(Schmidt et al., 1993), we sought its presence in activated
microglia in AD brain.   Microglia, identified by staining
with CD68 (Fig. 8A), proximate to a senile plaque, show
30 strong expression of RAGE antigen (Fig. 8B).

Radioligand binding studies with BV-2 cells demonstrated
dose-dependent binding of ^{125}I-Aß(1-40) with Kd ≈30nM (Fig.
9A).   Blockade of RAGE, with either excess sRAGE or
35 anti-RAGE IgG, prevented binding of ^{125}I-Aß to microglia,
although soluble vascular cell adhesion molecule-1 (another

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immunoglobulin superfamily molecule) or nonimmune rabbit IgG
was without effect (Fig. 9B).

        As RAGE appeared to have a central role in tethering Aß to
5 microglia, it was considered whether Aß-RAGE interaction
    might underlie Aß-induced migration of microglia. Using
    BV-2 cells, addition of Aß (1-40 or 25-35) to
    microchemotaxis chambers resulted in directional cell
    migration, dependent on a concentration gradient of the
10 stimulus from upper to lower chamber (Fig. 10A). Cell
    migration was prevented by addition of anti-RAGE F(ab')$_2$,
    whereas this reagent had no affect on motility of BV-2 cells
    following addition of formylated peptide
    (formyl-methionyl-leucinyl phenylalanine, fMLP)(Fig. 10B).
15 In contrast, nonimmune rabbit IgG was without effect on
    Aß-induced migration of BV-2 cells. Because of the known
    accumulation of microglia approximate to deposits of Aß, it
    was possible that, in contrast to soluble Aß, immobilized Aß
    might block microglial migration. To test this concept,
20 either Aß or albumin was adsorbed to the upper surface of
    chemotaxis chamber membranes and BV-2 cells were added; cell
    migration across Aß-coated membranes was diminished in
    response to a range of fMLP concentrations added to the
    lower compartment, whereas albumin-coated membranes allowed
25 free passage of cells (Fig. 10C), comparable to untreated
    membranes. Blockade of RAGE with anti-RAGE F(ab')$_2$ enhanced
    BV-2 migration across Aß-coated membranes to levels observed
    in membranes coated with albumin, whereas nonimmune F(ab')$_2$
    was without effect. Similar results were observed in a more
30 limited number of studies on microglia isolated from rat
    brain). These data indicate that soluble Aß induces
    microglial migration whereas immobilized Aß arrests
    microglial migration, in each case due to Aß engagement of
    RAGE.
35

    Activation of microglia, potentially producing cytotoxic

cytokines and other species (Dickson et al., 1993; McGeer et al., 1993; Meda et al., 1995; and Araujo et al., 1992), may be an important pathway to neuronal damage in AD. Of these, the ability of Aß to stimulate microglial expression of
5   tumor necrosis factor-a (TNFα) (Meda et al., 1995) may be especially significant, as TNFa could recruit additional host effector mechanisms. BV-2 cells incubated with Aß demonstrated induction of TNFα transcripts by PCR (Fig. 11A), followed by elaboration of TNFα into culture
10   supernatants (Fig. 11B). Blockade of RAGE, with anti-RAGE F(ab')$_2$, prevented the appearance of TNFα mRNA and diminished TNFα antigen to levels seen in untreated cells, whereas nonimmune F(ab')$_2$. A mechanism through which Aß and other cytokines influence cellular activation is by inducing
15   nuclear translocation of the transcription factor NF-kB, often utilizing an oxidant-mediated pathway (Schreck et al., 1991). Incubation of BV-2 cells with microglia induced NF-kB activation which was suppressed by blocking RAGE with anti-RAGE F(ab')$_2$ (Fig. 11C) or the antioxidant probucol.
20   Nonimmune F(ab')$_2$ was without effect.

These data show that RAGE has a pivotal role in the interaction of Aß with microglial cells. Soluble Aß binds RAGE and induces microglial migration along a concentration
25   gradient, likely to end at a deposit of insoluble Aß. Immobilized Aß (prepared as above or after allowing time for fibrils of Aß to form) prevents cell migration, permitting microglia to undergo the sustained activation which may underlie a chronic inflammatory component in AD(Rogers et
30   al., 1993; and Breitner et al., 1994). Indeed, in some studies it was found that immobilized Aß does induce microglial activation, and the time course for TNF production is more prolonged than that observed following exposure of microglia to soluble Aß. Although these data
35   indicate that RAGE is important in mediating microglial binding and subsequent cellular activation following

exposure to Aß, in view of the number of different polypeptides with which Aß has been shown to interact (Iversen et al., 1995), it is likely that other proteins will also be identified which participate with RAGE in
5   Aß-mediated cellular activation.   In this context, these studies have emphasized the potential of Aß-RAGE interaction to activate microglia, accelerating events leading to neurodegeneration.   However, RAGE processing of Aß (i.e., endocytosis and degradation), under conditions where
10 substantial amounts of Aß are present probably represents a protective mechanism gone awry: it is possible that small amounts of Aß might be endocytosed and degraded by RAGE-dependent mechanisms, whereas larger amounts of peptide might overwhelm this mechanism and result in Aß-RAGE-induced
15 cellular activation.   These studies complement the recent observation that RAGE is expressed at higher levels in neurons approximate to Aß deposits (Yan et al., 1996).   RAGE appears to mediate oxidant stress and neurotoxicity, as shown by their attenuation following blockade of the
20 receptor (Yan et al., 1996).   The possibility that Aß in AD is subject to nonenzymatic glycation with formation of advanced glycation endproducts (the latter were the first recognized ligands of RAGE) provides an additional mechanism for enhancing its interaction with RAGE (Vitek et al.,
25 1994).   Taken together, these observations suggest that increased expression of RAGE in AD brain may have a central role in Aß-mediated microglial activation, as well as neuronal perturbation.   Thus, interfering with Aß-RAGE interaction might delay the development of subsequent
30 cellular events resulting in neuronal dysfunction.

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20

-48-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Stern, David
                Schmidt, Ann Marie
                Yan, Shi Du

    (ii) TITLE OF INVENTION: A POLYPEPTIDE FROM LUNG EXTRACT WHICH
         BINDS AMYLOID-BETA PEPTIDE

   (iii) NUMBER OF SEQUENCES: 10

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    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE: 26-JAN-1996
        (C) CLASSIFICATION:

   (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: White, John P.
        (B) REGISTRATION NUMBER: 28,678
        (C) REFERENCE/DOCKET NUMBER: 48316

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 212-278-0400
        (B) TELEFAX: 212-391-0526

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 4 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

    Arg Gly Asp Ser
    1

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:

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                    (A) LENGTH: 40 amino acids
                    (B) TYPE: amino acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: peptide




            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

            Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
            1               5                   10              .       15

            Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
                        20          25                  30

            Gly Leu Met Val Gly Gly Val Val
                    35              40
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(2) INFORMATION FOR SEQ ID NO:3:

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          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: peptide

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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            Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met
            1               5                   10
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(2) INFORMATION FOR SEQ ID NO:4:

      (i) SEQUENCE CHARACTERISTICS:
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          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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22
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(2) INFORMATION FOR SEQ ID NO:5:

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          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

-50-

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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25

(2) INFORMATION FOR SEQ ID NO:6:

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          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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22

(2) INFORMATION FOR SEQ ID NO:7:

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          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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24

(2) INFORMATION FOR SEQ ID NO:8:

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          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: peptide

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

      Asp Gln Asn Ile Thr Ala Arg Ile Gly Lys Pro Leu Val Leu Asn Cys
      1   5   10   15

Lys Gly Ala Pro Lys Lys Pro Pro Gln Gln Leu Glu Trp Lys
         20   25   30

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

    Asp Gln Xaa Ile Thr Ala Arg Ile Gly Lys Pro Leu Val Leu Asn Xaa
    1   5   10   15

    Lys Gly Ala Pro Lys Lys Pro Pro Gln Gln Leu Glu Trp Lys
             20   25   30

(2) INFORMATION FOR SEQ ID NO:10:

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        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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    1   5   10   15

    Lys Gly Ala Pro
             20

What is claimed is:

1.   A method for inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which comprises contacting the cell with an agent capable of inhibiting interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an amount effective to inhibit interaction of the amyloid-β peptide with the receptor for advanced glycation end product on the surface of the cell.

2.   The method of claim 1, wherein the cell is a neuronal cell, an endothelial cell, a glial cell, a microglial cell, a smooth muscle cell, a somatic cell, a bone marrow cell, a liver cell, an intestinal cell, a germ cell, a myocyte, a mononuclear phagocyte, an endothelial cell, a tumor cell, or a stem cell.

3.   The method of claim 1, wherein the agent is a peptide, a peptidomimetic, a nucleic acid or a small molecule.

4.   The method of claim 3, wherein the peptide comprises at least a portion of the sequence -Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val- (Seq. I.D. No. 2).

5.   The method of claim 3, wherein the peptide comprises at least a portion of the sequence -Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met- (Seq. I.D. No. 3).

6.   The method of claim 1, wherein the agent is soluble receptor for advanced glycation end product.

7.   The method of claim 1, wherein the agent is a soluble

extracellular portion of a receptor for advanced glycation end product.

8. The method of claim 1, wherein the agent is an antibody or portion thereof.

9. The method of claim 8, wherein the antibody is capable of specifically binding to the receptor for advanced glycation endproduct.

10. The method of claim 8, wherein the antibody is a monoclonal antibody.

11. The method of claim 8, wherein the antibody is a polyclonal antibody.

12. The method of claim 8, wherein the portion of the antibody comprises a Fab.

13. The method of claim 8, wherein the portion of the antibody comprises a complementarity determining region or a variable region.

14. The method of claim 1, wherein the agent is capable of specifically binding to the amyloid-β peptide.

15. The method of claim 14, wherein the agent binds to the amyloid-β peptide at the site where the receptor for advanced glycation end product interacts.

16. A method for inhibiting degeneration of a neuronal cell which comprises contacting the cell with an agent capable of inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of the neuronal cell according to the method of claim 1.

17. A method for inhibiting formation of an amyloid-β
     peptide fibril on a cell which comprises contacting the
     cell with an agent capable of inhibiting interaction of

5   an amyloid-β peptide with a receptor for advanced
     glycation end product on the surface of the cell
     according to the method of claim 1.

18. A method for inhibiting extracellular assembly of an

10   amyloid-β peptide into a fibril which comprises
     contacting the amyloid-β peptide with an agent capable
     of inhibiting interaction of an amyloid-β peptide with
     another amyloid-β peptide according to the method of
     claim 1.

15

19. A method for inhibiting aggregation of amyloid-β
     peptide on the surface of a cell which comprises
     contacting the amyloid-β peptide with an agent capable
     of inhibiting interaction of the amyloid-β peptide with

20   a receptor for advanced glycation end product according
     to the method of claim 1.

20. A method for inhibiting aggregation of amyloid-β
     peptide on the surface of a cell which comprises

25   contacting the receptor for advanced glycation end
     product with an agent capable of inhibiting interaction
     of the amyloid-β peptide with the receptor for advanced
     glycation end product according to the method of claim
     1.

30

21. A method for inhibiting infiltration of a microglial
     cell into senile plaques which comprises contacting the
     microglial cell with an agent capable of inhibiting
     interaction of an amyloid-β peptide with a receptor for

35   advanced glycation end product on the surface of the
     microglial cell according to the method of claim 1.

22.   A method for inhibiting activation of a microglial cell by an amyloid-β peptide which comprises contacting the microglial cell with an agent capable of inhibiting

5   interaction of the amyloid-β peptide with a receptor for advanced glycation end product on the surface of the microglial cell according to the method of claim 1.

23.   The method of claim 22, wherein the inhibition of

10   activation comprises decreased production of cytokines by the microglial cell.

24.   The method of claim 1, wherein the interaction is binding of the amyloid-β peptide to the receptor for

15   advanced glycation end product on the surface of the cell.

25.   A method for treating a subject with a condition associated with interaction of an amyloid-β peptide

20   with a receptor for advanced glycation end product on a cell, which comprises administering to the subject an agent capable of inhibiting the interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an

25   amount effective to inhibit the amyloid-β peptide interaction with the receptor for advanced glycation end product on the cell thereby treating the subject.

26.   The method of claim 25, wherein the condition is

30   diabetes, Alzheimer's Disease, senility, renal failure, hyperlipidemic atherosclerosis, neuronal cytotoxicity, Down's syndrome, dementia associated with head trauma, amyotrophic lateral sclerosis, multiple sclerosis or neuronal degeneration.

35

27.   The method of claim 25, wherein the subject is a

mammal.

28. The method of claim 27, wherein the mammal is a human.

5 29. The method of claim 25, wherein the administration comprises intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; topical, nasal, oral, anal, ocular or otic delivery.

10

30. The method of claim 25, wherein the agent is soluble receptor for advanced glycation end product.

31. The method of claim 25, wherein the agent is a soluble
15 extracellular portion of a receptor for advanced glycation end product.

32. The method of claim 25, wherein the agent is a peptide, a peptidomimetic, a nucleic acid or a small molecule.

20

33. The method of claim 32, wherein the peptide comprises at least a portion of the sequence -Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-
25 Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val- (Seq. I.D. No. 2).

34. The method of claim 32, wherein the peptide comprises at least a portion of the sequence -Gly-Ser-Asn-Lys-
30 Gly-Ala-Ile-Ile-Gly-Leu-Met- (Seq. I.D. No. 3).

35. The method of claim 25, wherein the agent is an antibody or portion thereof.

35 36. The method of claim 35, wherein the antibody is capable of specifically binding to the receptor for advanced

glycation endproduct.

37. The method of claim 35, wherein the antibody is a monoclonal antibody.

38. The method of claim 35, wherein the antibody is a polyclonal antibody.

39. The method of claim 35, wherein the portion of the antibody comprises a Fab.

40. The method of claim 35, wherein the portion of the antibody comprises a complementarity determining region or a variable region.

41. The method of claim 25, wherein the agent is capable of specifically binding to the amyloid-β peptide.

42. The method of claim 41, wherein the agent binds to the amyloid-β peptide at the site where the receptor for advanced glycation end product interacts.

43. The method of claim 25, wherein the condition is associated with degeneration of a neuronal cell in the subject.

44. The method of claim 25, wherein the condition is associated with formation of an amyloid-β peptide fibril.

45. The method of claim 25, wherein the condition is associated with aggregation of amyloid-β peptide.

46. The method of claim 25, wherein the condition is associated with infiltration of a microglial cell into a senile plaque.

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47. The method of claim 25, wherein the condition is associated with activation of a microglial cell by an amyloid-β peptide.

48. The method of claim 47, wherein the activation comprises production of cytokines by the microglial cell.

49. A method for evaluating the ability of an agent to inhibit binding of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which comprises:

   (a) contacting the cell with the agent and amyloid-β peptide;

   (b) determining the amount of amyloid-β peptide bound to the cell, and

   (c) comparing the amount of bound amyloid-β peptide determined in step (b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit the binding of amyloid-β peptide to the receptor for advanced glycation end product on the surface of the cell.

50. The method of claim 49, wherein the cell is contacted with the agent and the amyloid-β peptide simultaneously.

51. The method of claim 49, wherein the cell is contacted with the amyloid-β peptide and the agent.

52. The method of claim 49, wherein the cell is a neuronal cell, an endothelial cell, a glial cell, a microglial

cell, a smooth muscle cell, a somatic cell, a bone
marrow cell, a liver cell, an intestinal cell, a germ
cell, a myocyte, a mononuclear phagocyte, an
endothelial cell, a tumor cell, or a stem cell.

53. The method of claim 49, wherein the agent is a peptide,
a peptidomimetic, a nucleic acid or a small molecule.

54. The method of claim 53, wherein the peptide comprises
at least a portion of the sequence -Asp-Ala-Glu-Phe-
Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-
Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-
Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val- (Seq. I.D. No.
2).

55. The method of claim 53, wherein the peptide comprises
at least a portion of the sequence -Gly-Ser-Asn-Lys-
Gly-Ala-Ile-Ile-Gly-Leu-Met- (Seq. I.D. No. 3).

56. The method of claim 49, wherein the agent is soluble
receptor for advanced glycation end product.

57. The method of claim 49, wherein the agent is a soluble
extracellular portion of receptor for advanced
glycation end product.

58. The method of claim 49, wherein the agent is an
antibody or portion thereof.

59. The method of claim 58, wherein the antibody is capable
of specifically binding to the receptor for advanced
glycation endproduct.

60. The method of claim 58, wherein the antibody is a
monoclonal antibody.

61. The method of claim 58, wherein the antibody is a polyclonal antibody.

62. The method of claim 58, wherein the portion of the antibody comprises a Fab.

63. The method of claim 58, wherein the portion of the antibody comprises a complementarity determining region or a variable region.

64. The method of claim 49, wherein the agent is capable of specifically binding to the amyloid-β peptide.

65. The method of claim 64, wherein the agent binds to amyloid-β peptide at the site where the receptor for advanced glycation end product interacts.

66. The method of claim 49, wherein the agent is a soluble extracellular portion of a receptor for advanced glycation end product.

67. The method of claim 49, wherein the agent is bound to a solid support.

68. The method of claim 49, wherein the agent is expressed on the surface of a cell.

69. A method for inhibiting activation of an NF-κB gene in a cell which comprises contacting the cell with an agent which is capable of inhibiting interaction of amyloid-β peptide with a receptor for advanced glycation endproduct on the cell, thus inhibiting activation of NF-κB in the cell.

70. A pharmaceutical composition which comprises an agent capable of inhibiting interaction of amyloid-β peptide

with a receptor for advanced glycation endproduct and
a pharmaceutically acceptable carrier.

71.   The pharmaceutical composition of claim 70, wherein the
5   carrier is a diluent, an aerosol, a topical carrier, an
       aquous solution, a nonaqueous solution or a solid
       carrier.

FIG. 1A   FIG. 1B   FIG. 1C

FIG. 1D   FIG. 1E   FIG. 1F
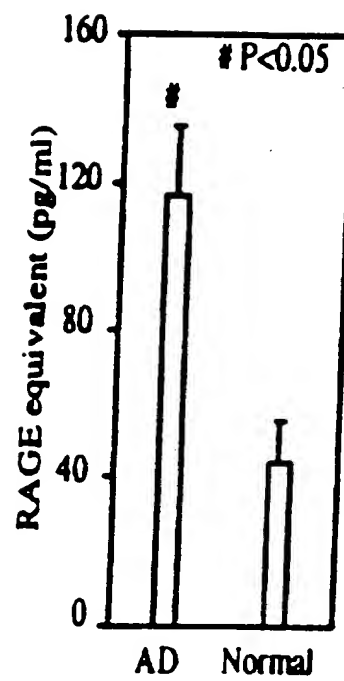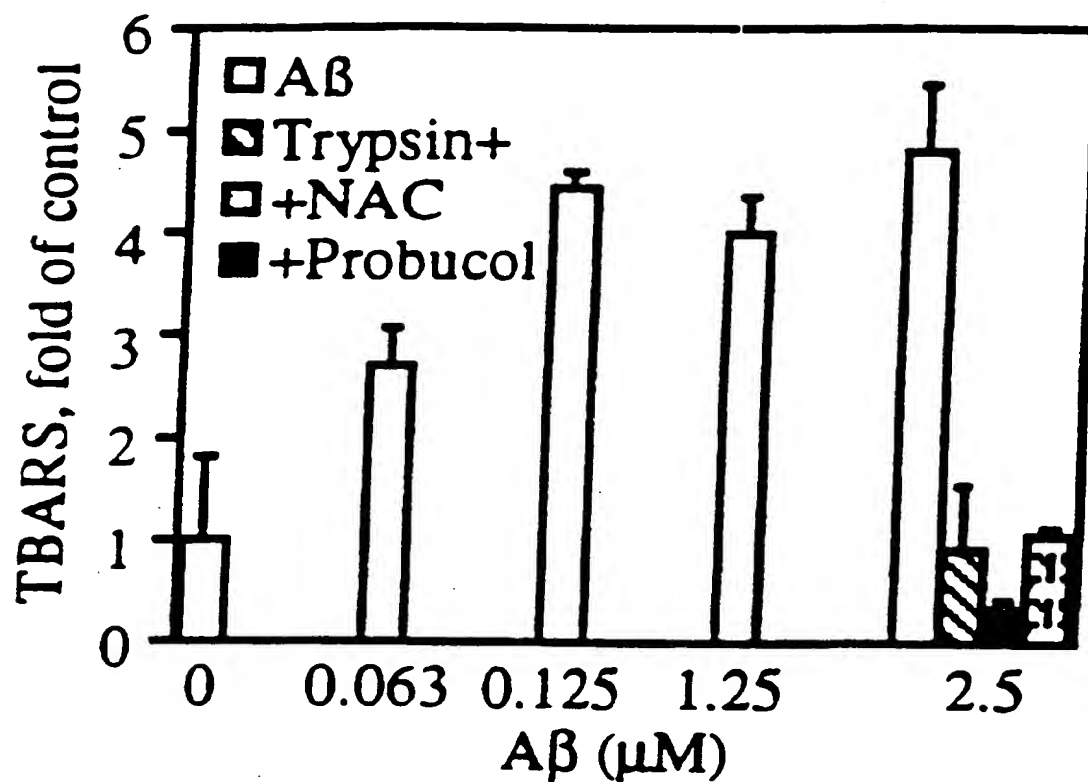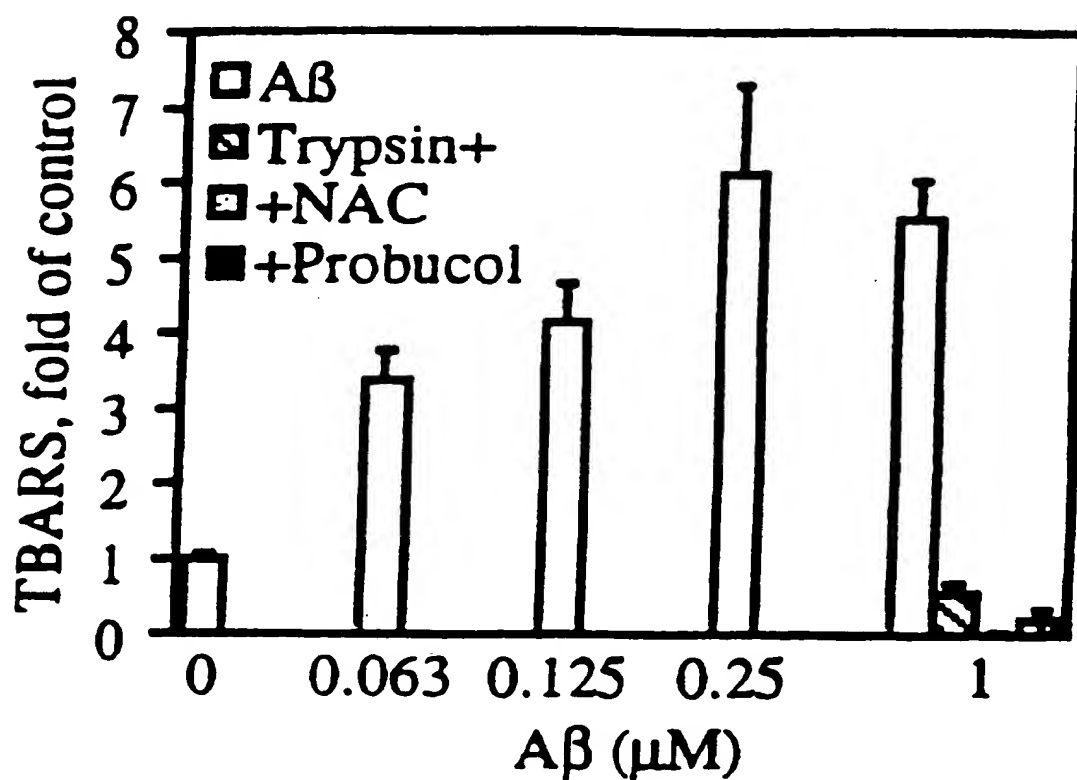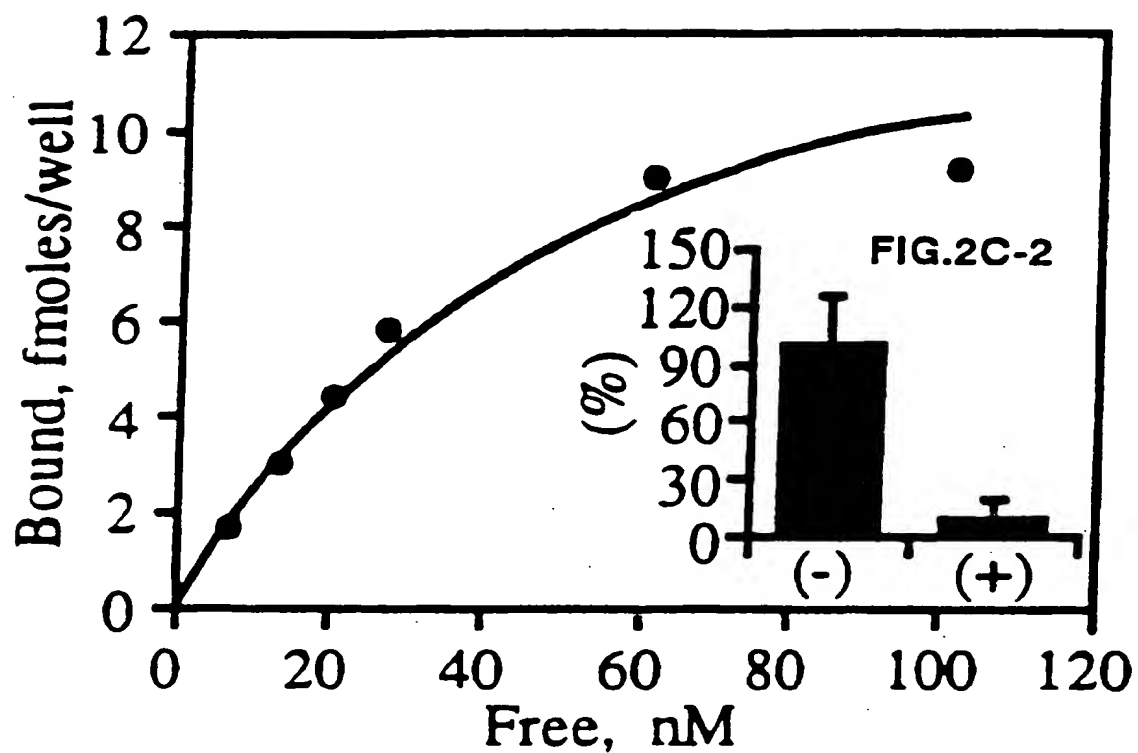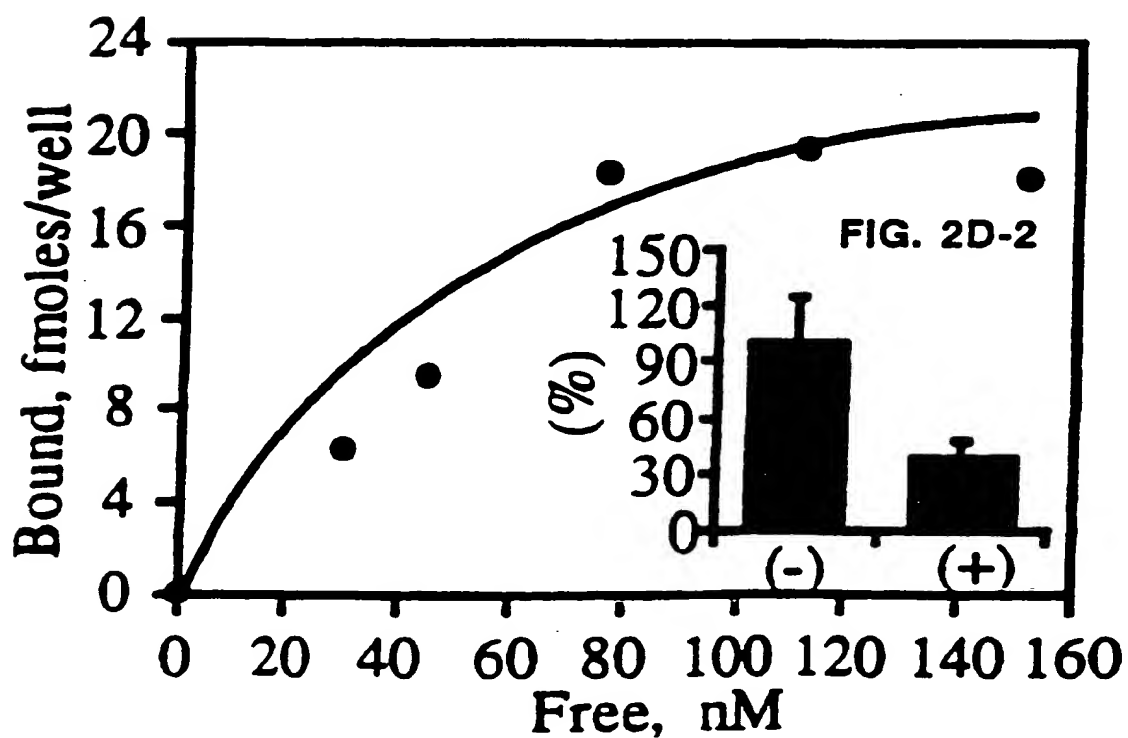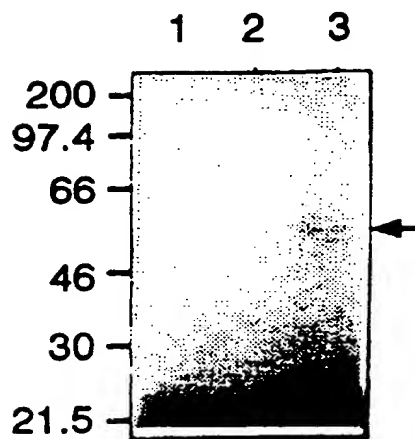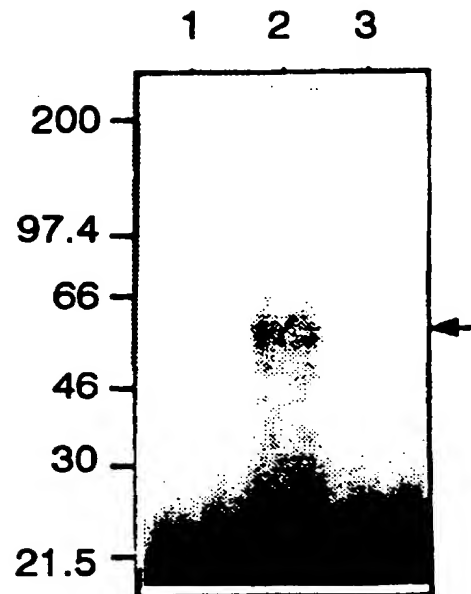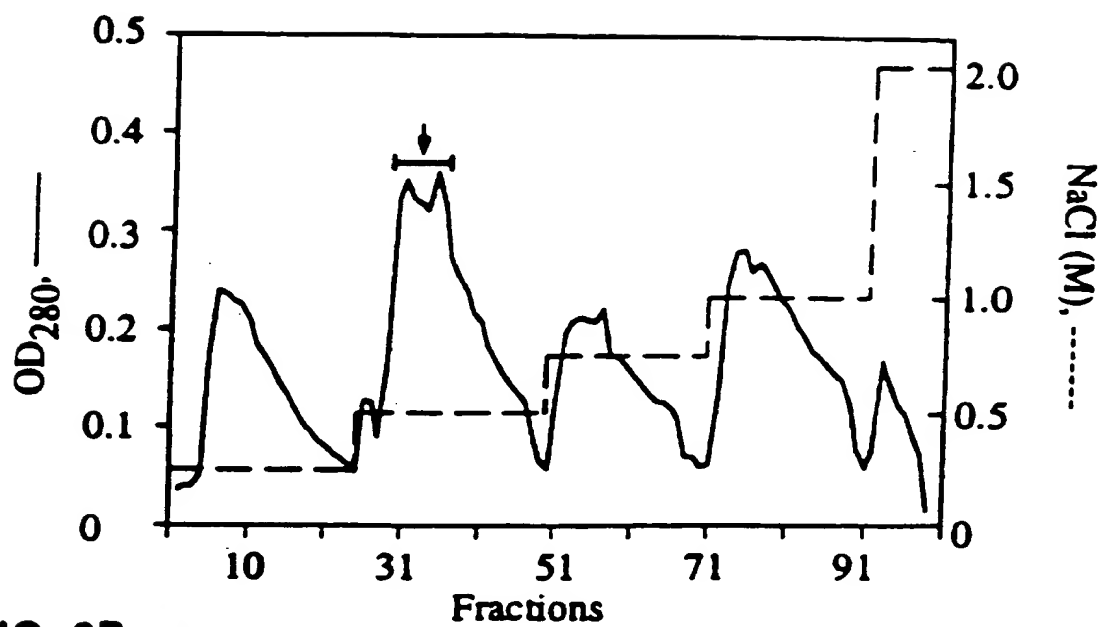
FIG. 1G

FIG. 2A

FIG. 2B

FIG. 2C-1

FIG.2C-2

FIG. 2D-1

FIG. 2D-2

FIG. 2E

FIG. 2F

FIG. 3A

FIG. 3B

FIG. 3C

^{125}I-Aβ Binding (cpm)

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

FIG. 4E

FIG. 5A

FIG. 5B

FIG. 5C

FIG. 6A

FIG. 6B

FIG. 6C

1 2 3 4 5 6

FIG. 6D

1 2 3 4 5 6
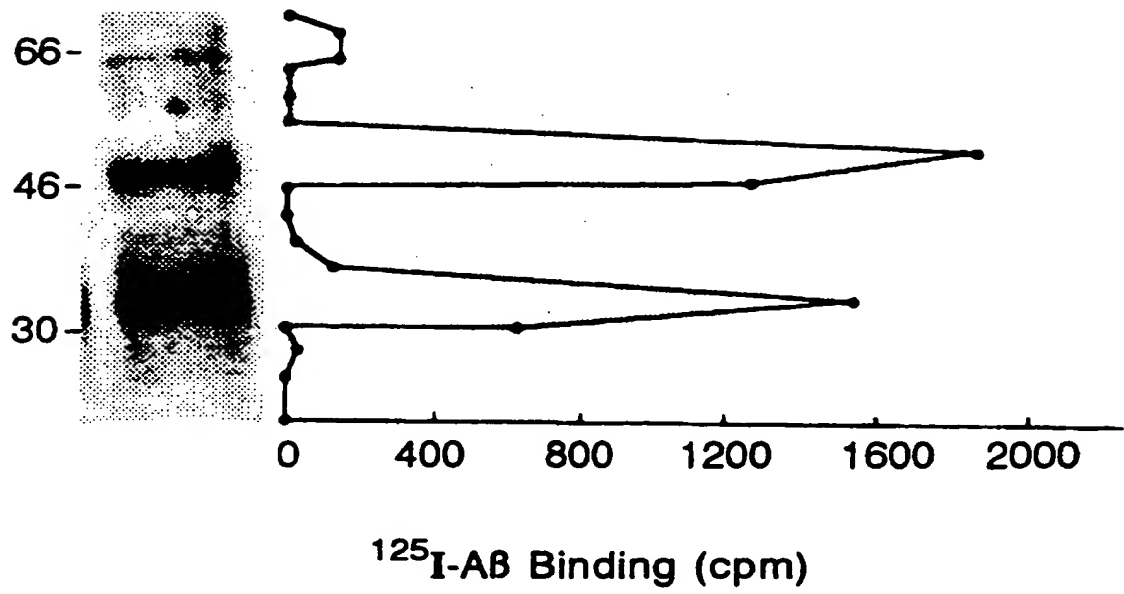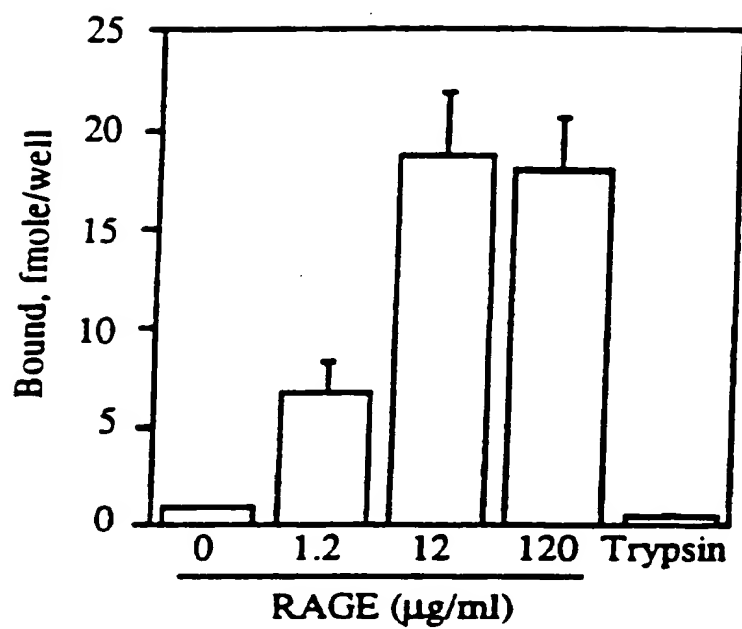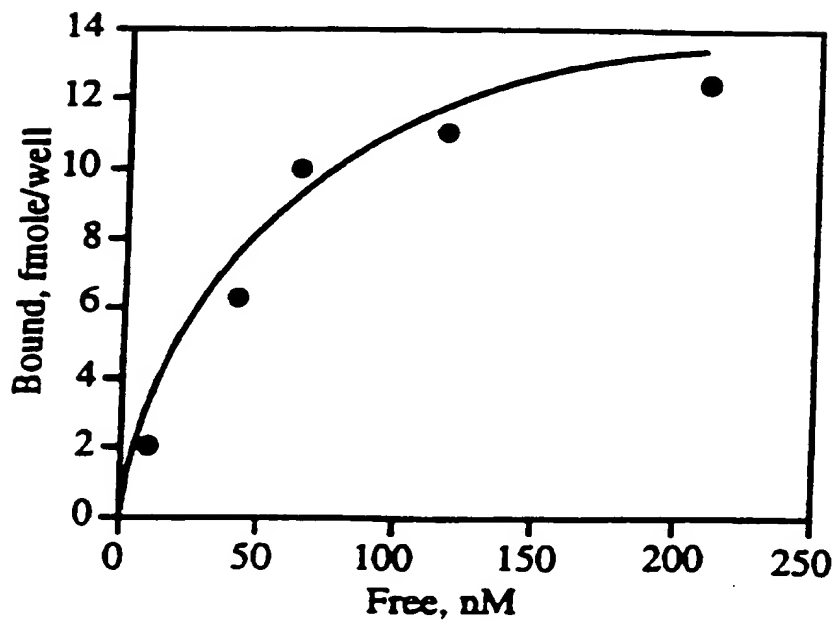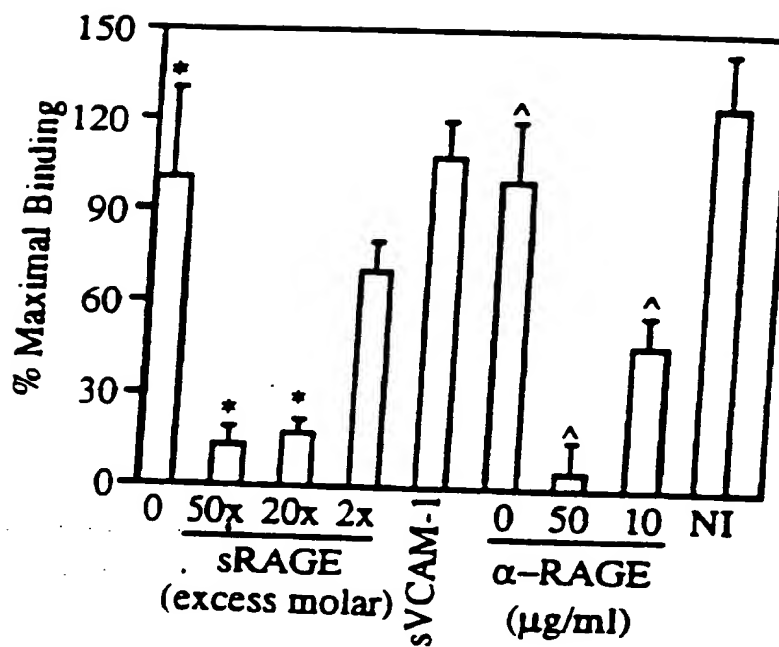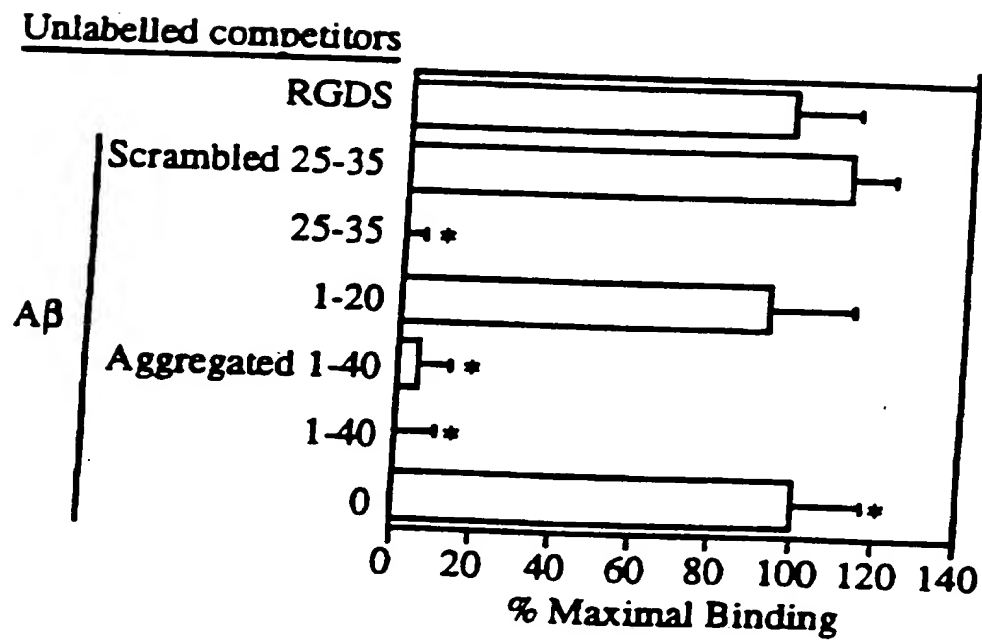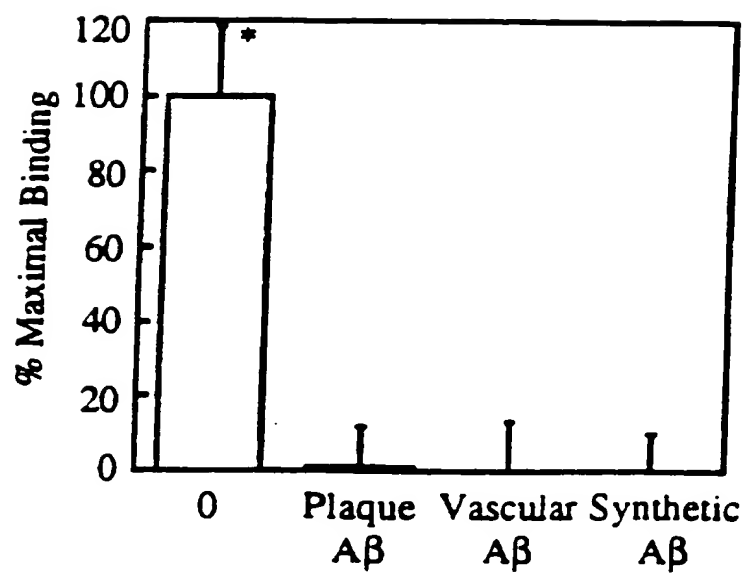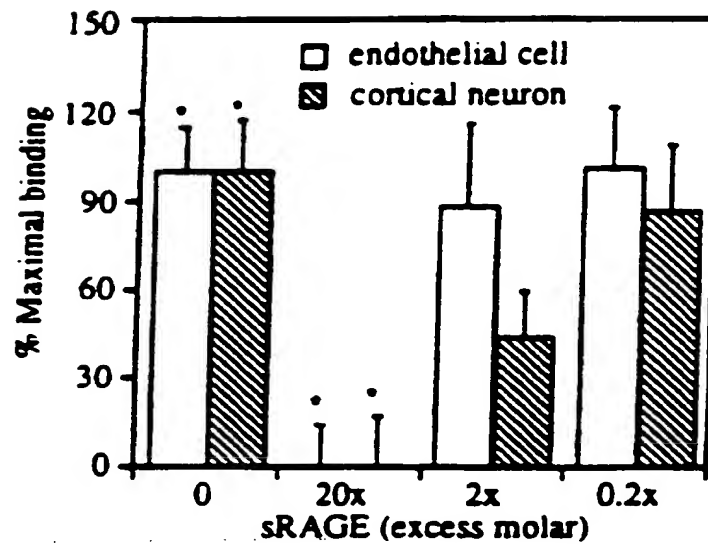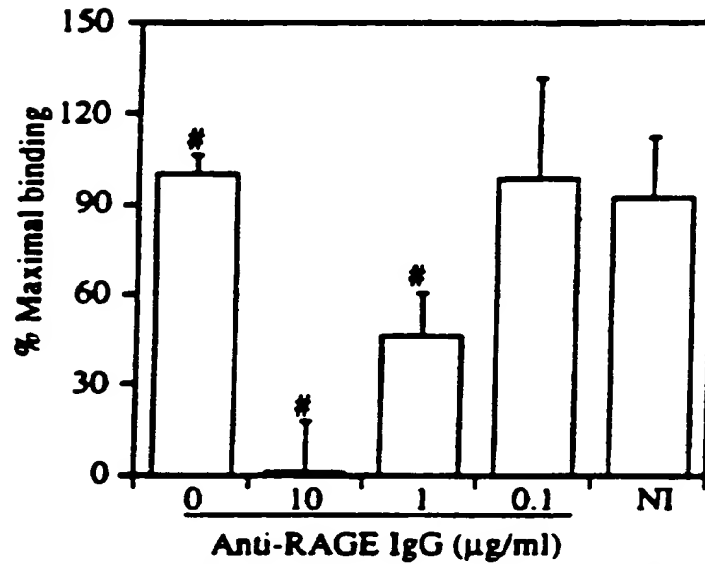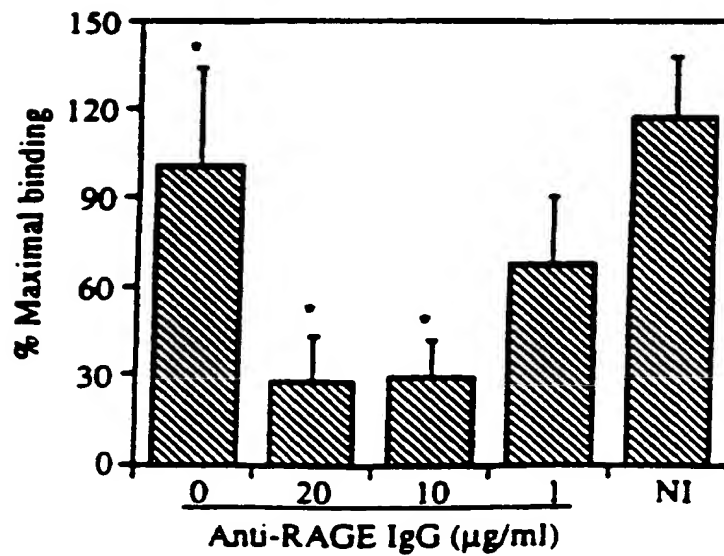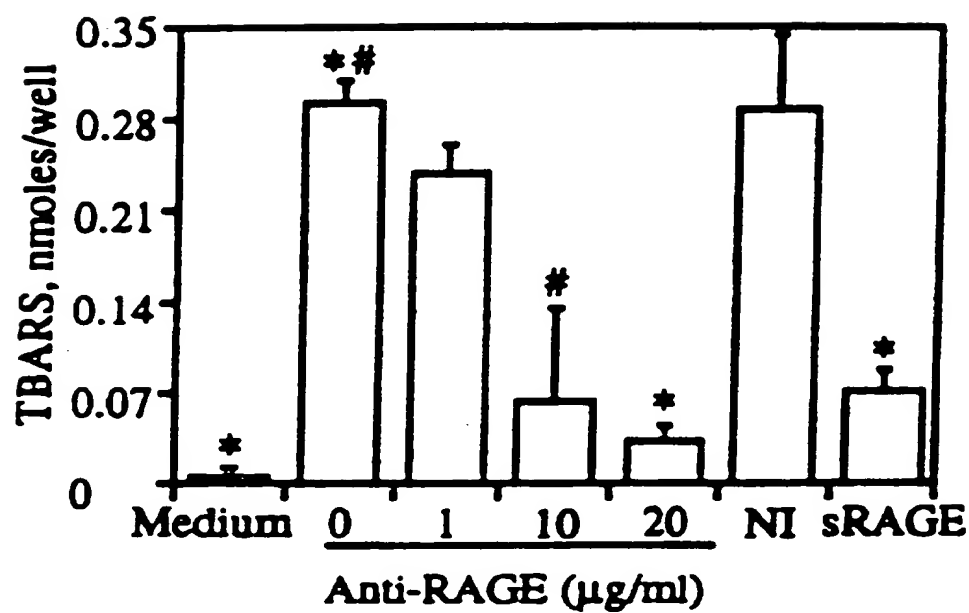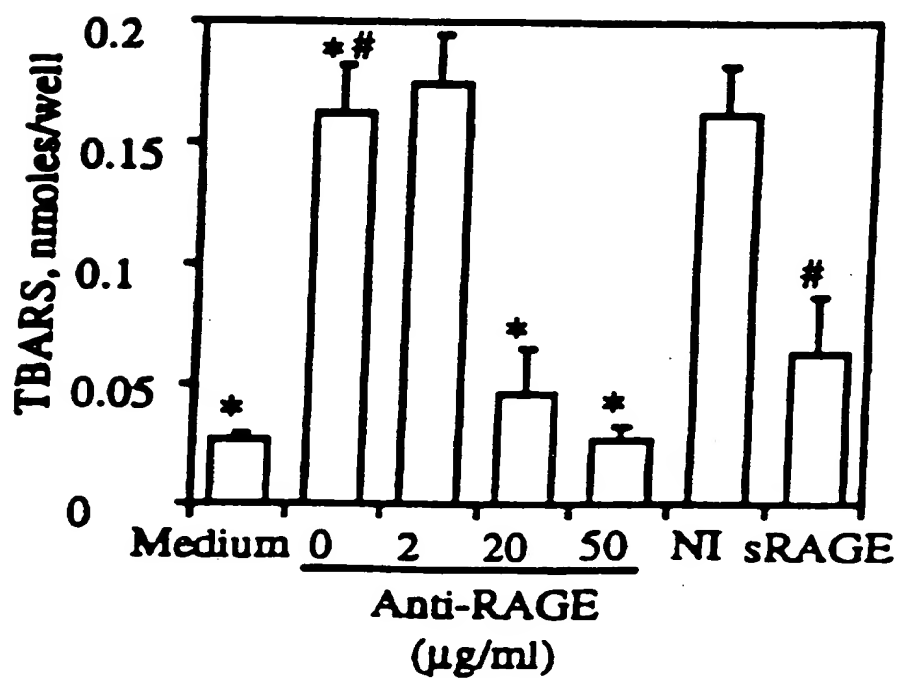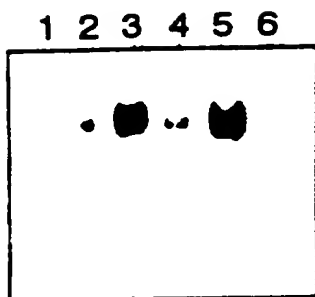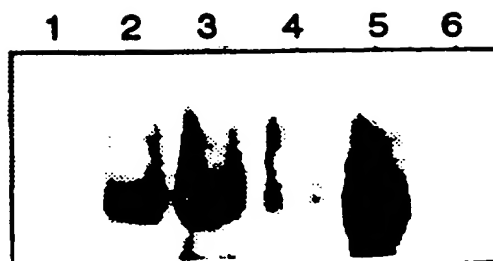
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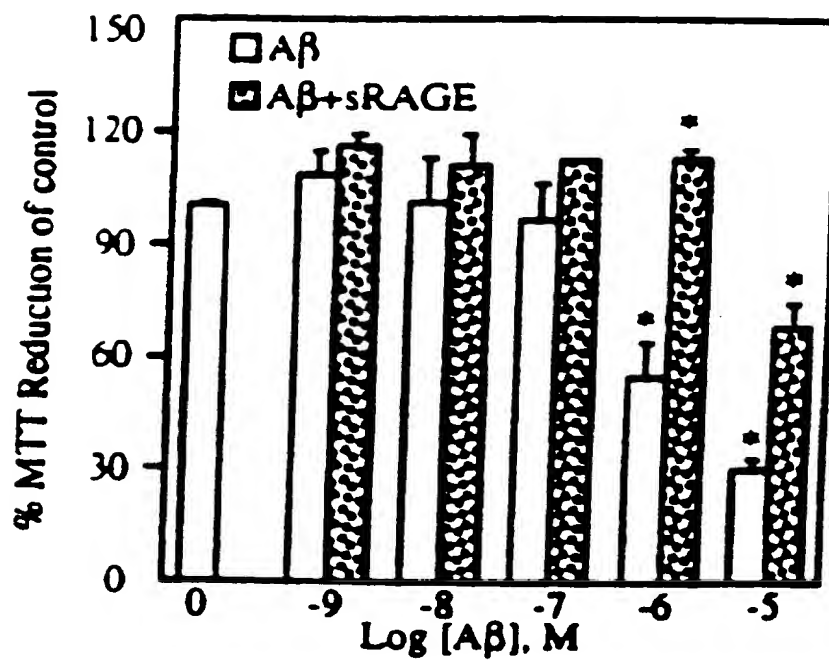
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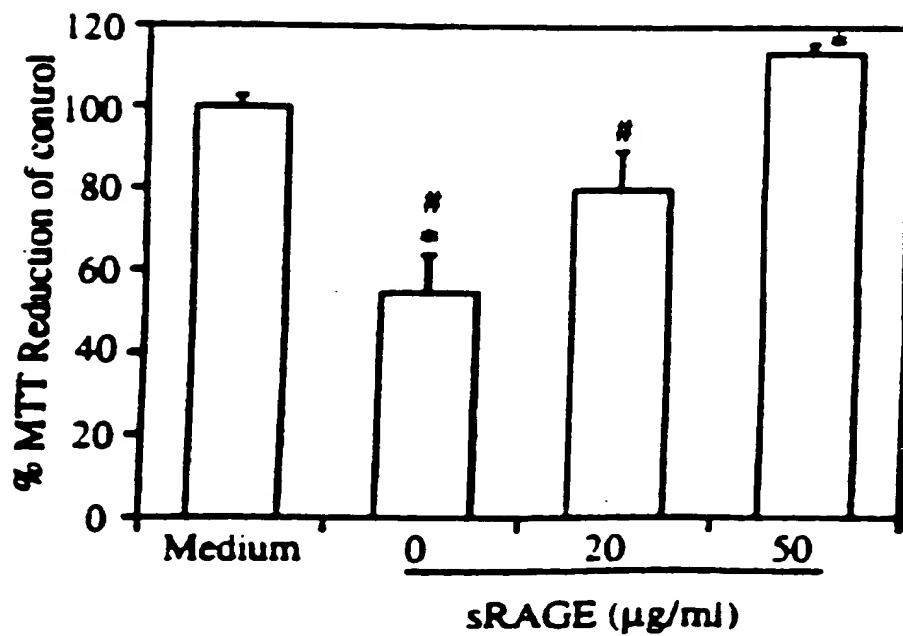
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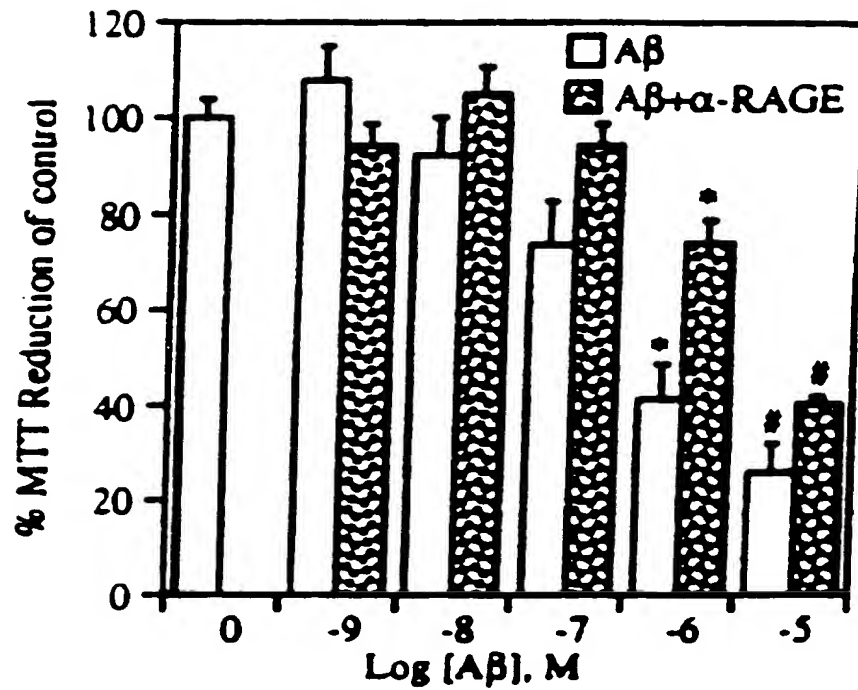
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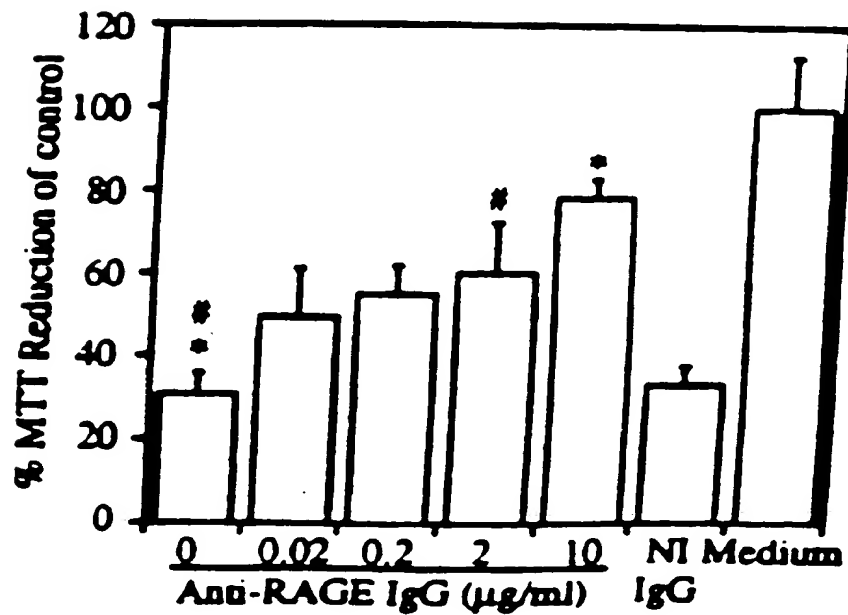
FIG. 7E　　　　　　　　　FIG. 7F

FIG. 7G　　　　　　　　　FIG. 7H

FIG. 8B
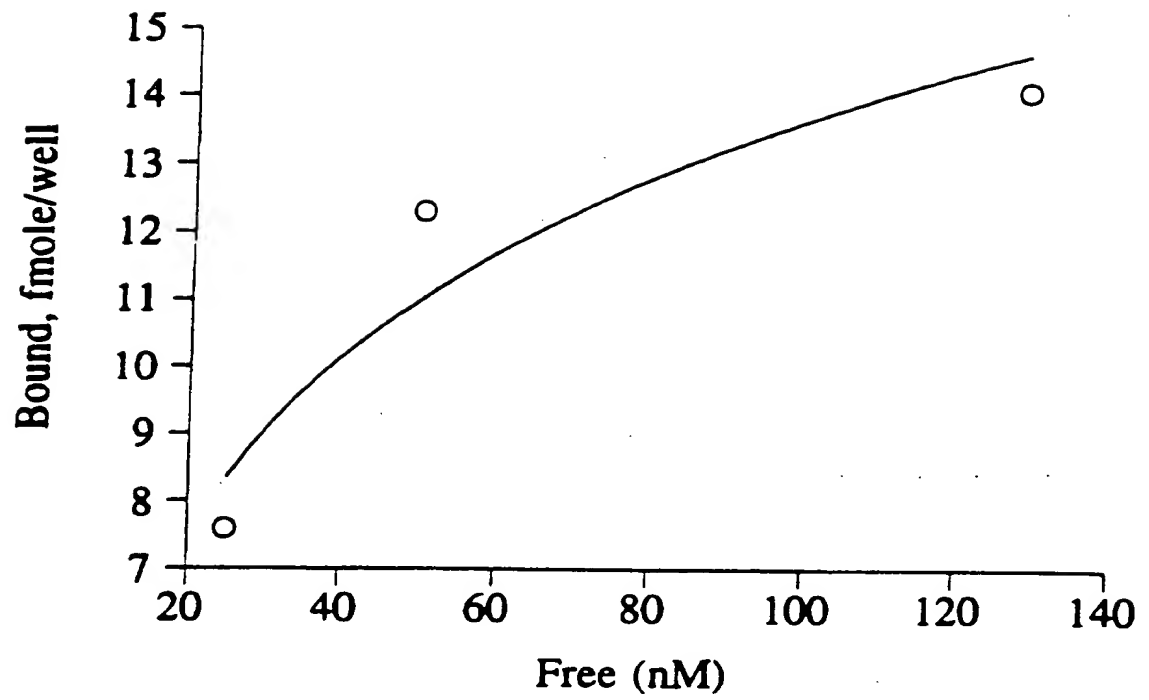
FIG. 8A

RAGE
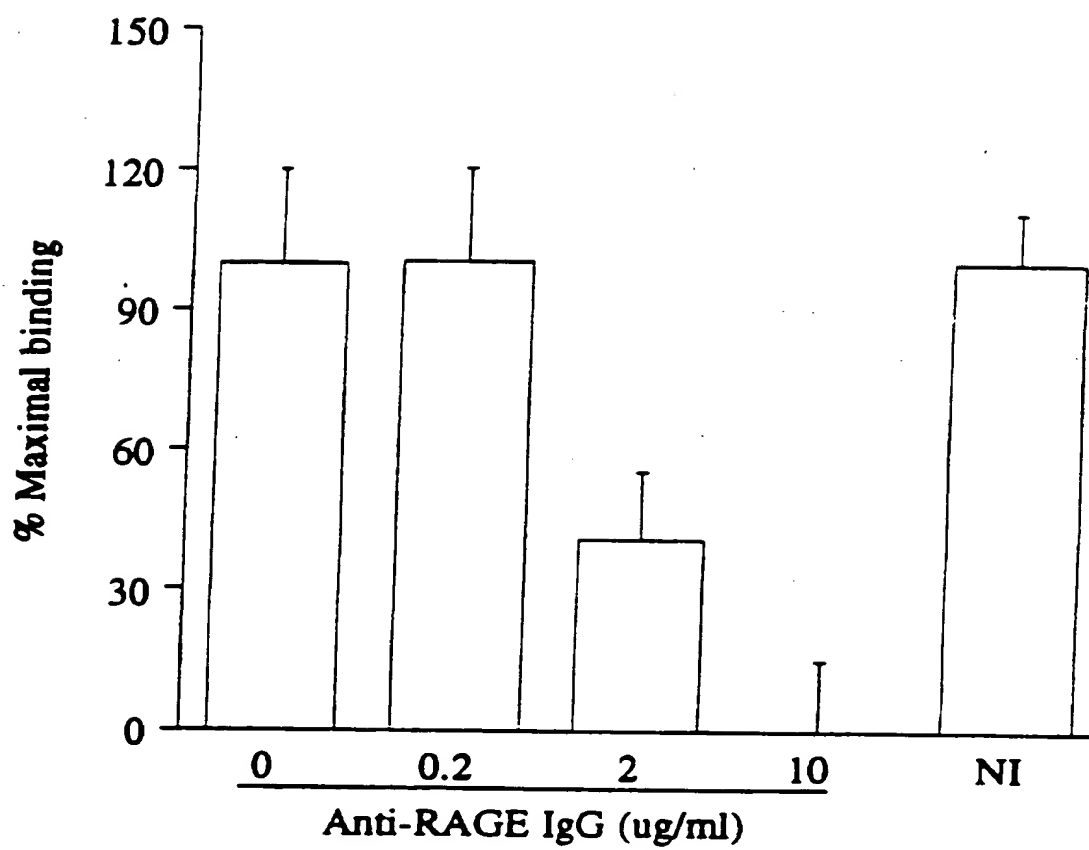
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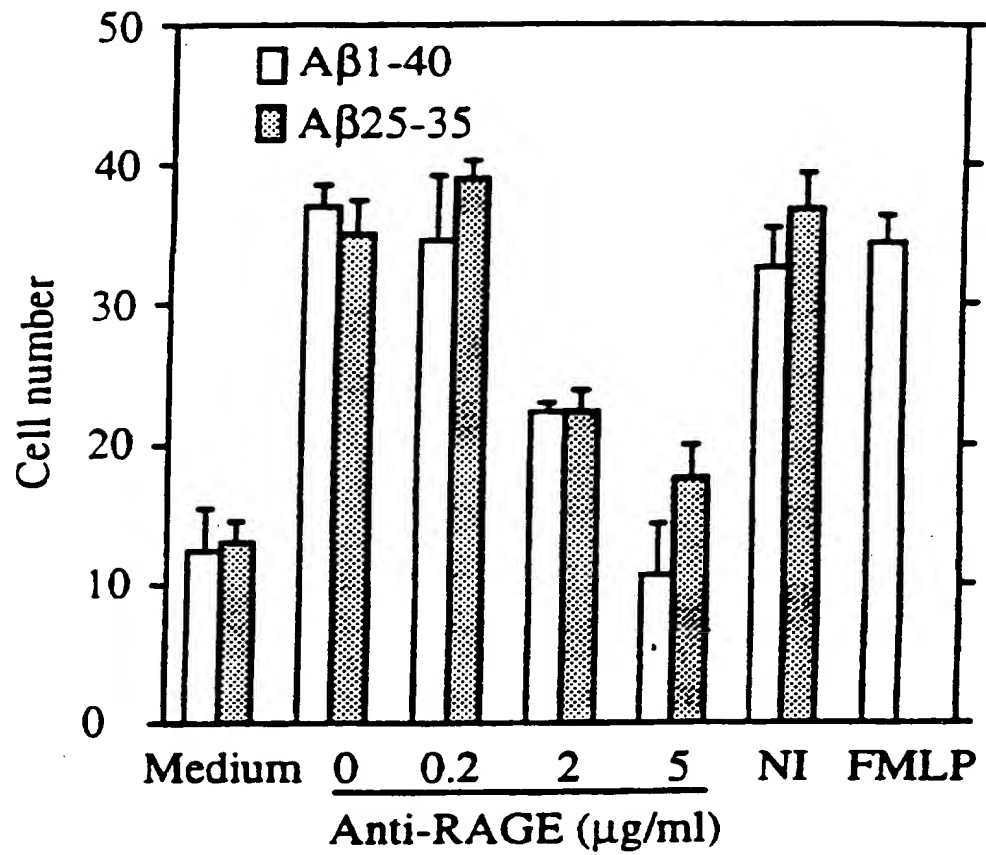
B

A

FIG. 9A

FIG. 9B

FIG. 10A

FIG. 10B

FIG. 10C

*P<0.01
#P<0.05

Cell number (y-axis, 0 to 40)

Anti-RAGE (ug/ml): 0, 0.2, 2, 5, 10, NI

Aβ adsorbed to upper surface of membrane

0, FMLP

Albumin adsorbed to upper surface of membrane
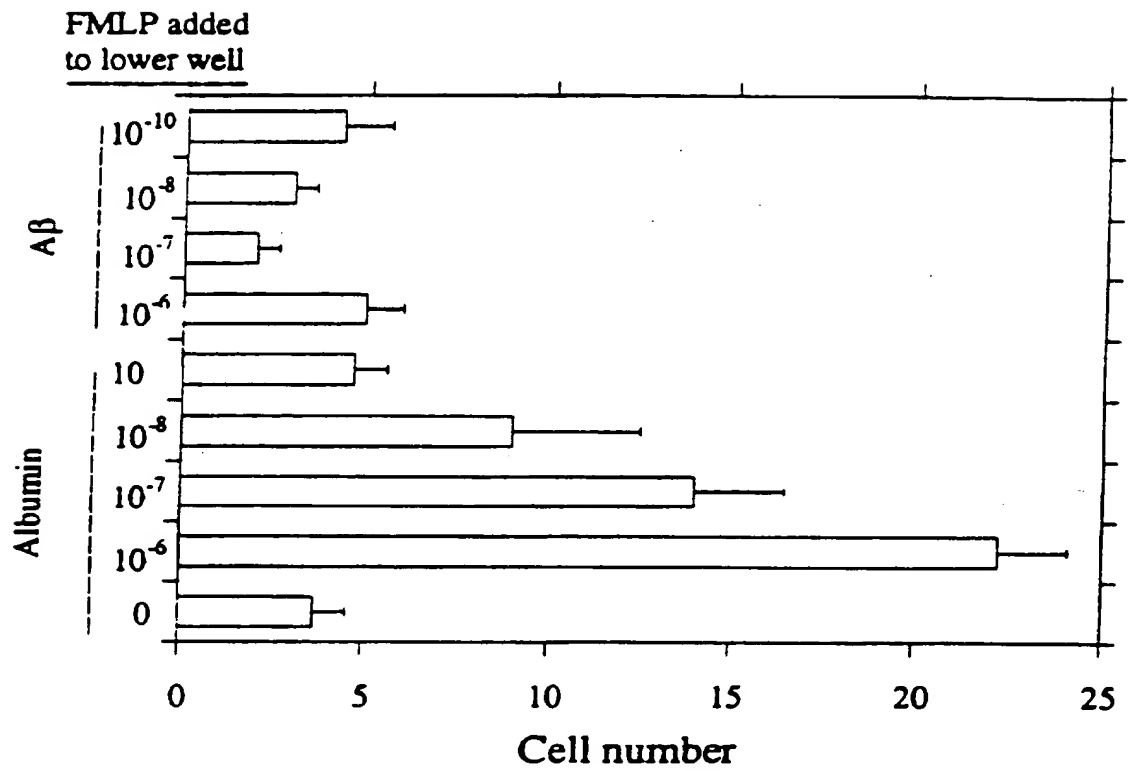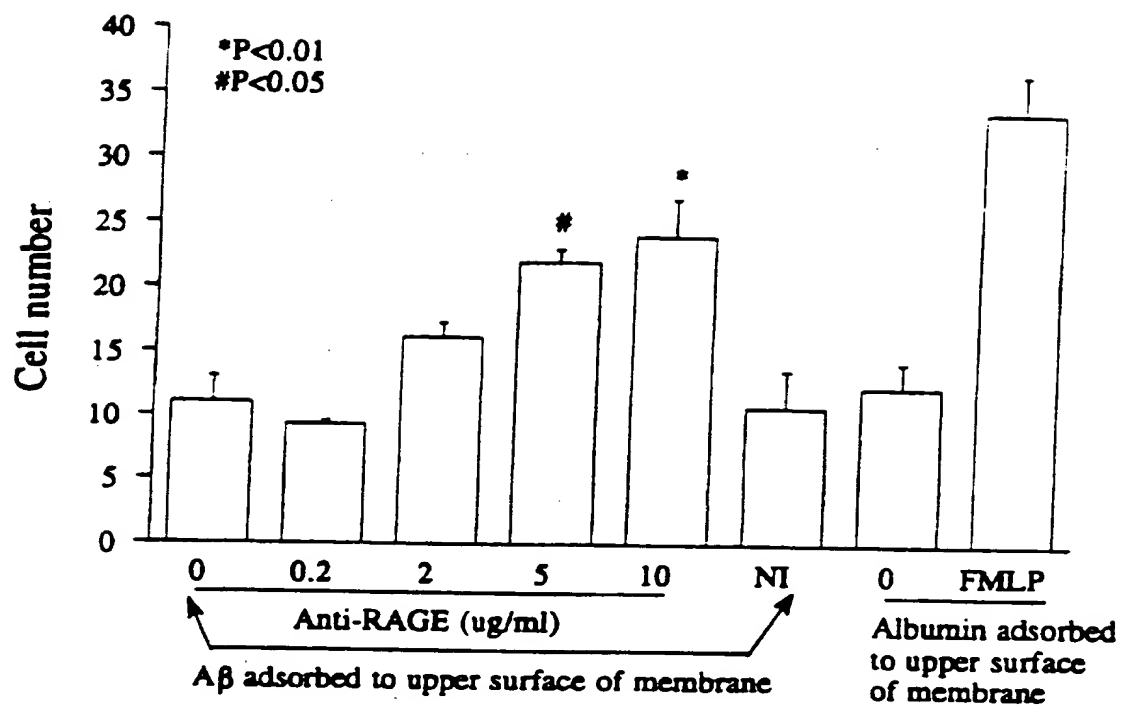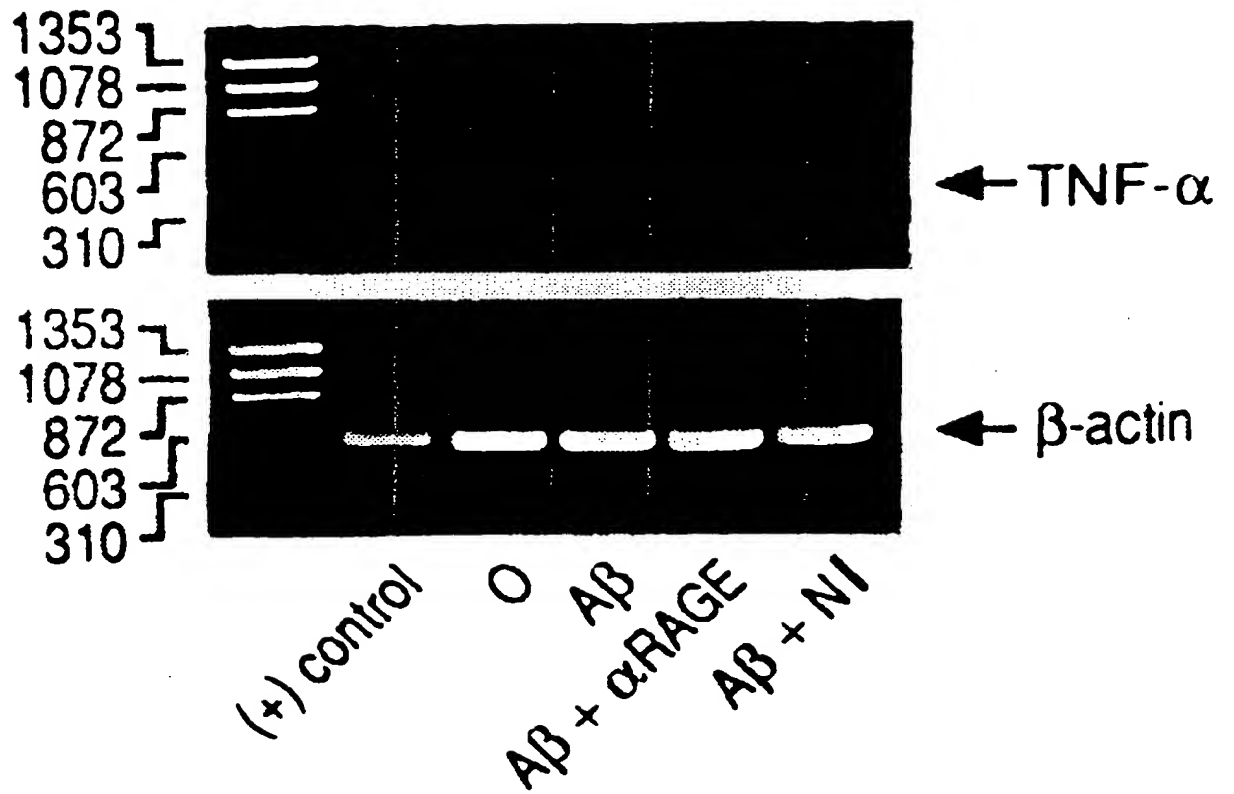
FIG. 11A

FIG. 11B

FIG. 11C

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   : A61K 39/395; A61K 38/00; C07K16/00
US CL   : 424/130.1; 514/2, 44; 530/300, 387.1
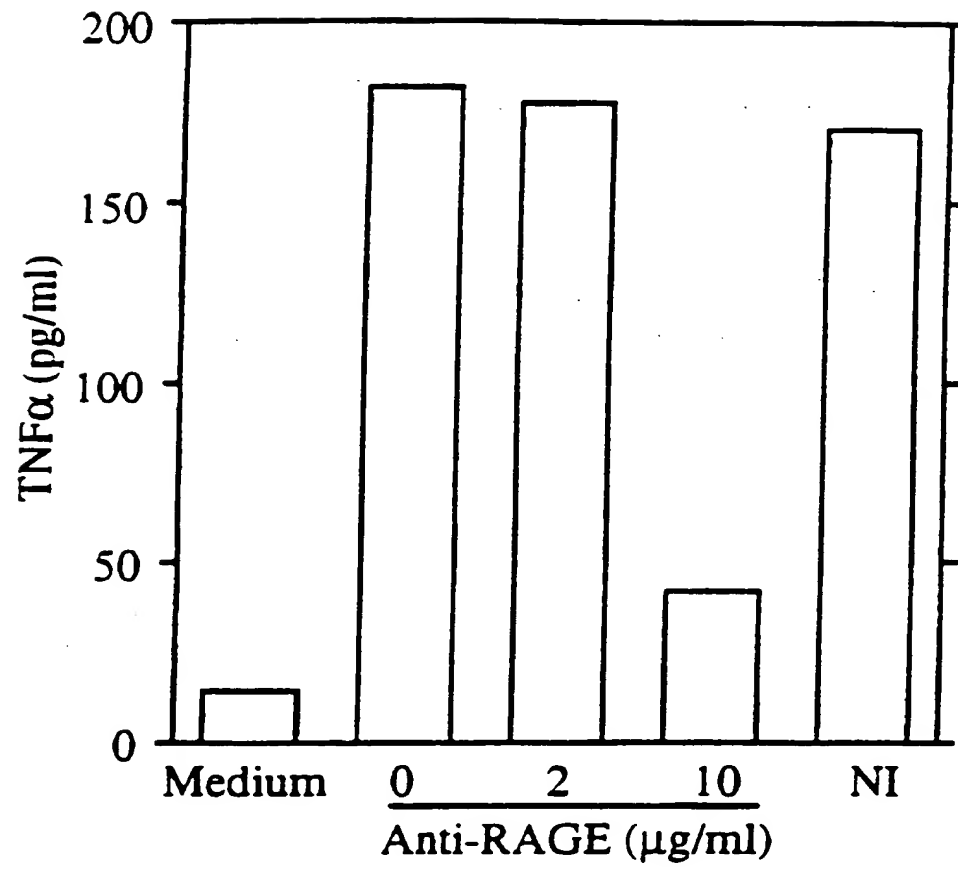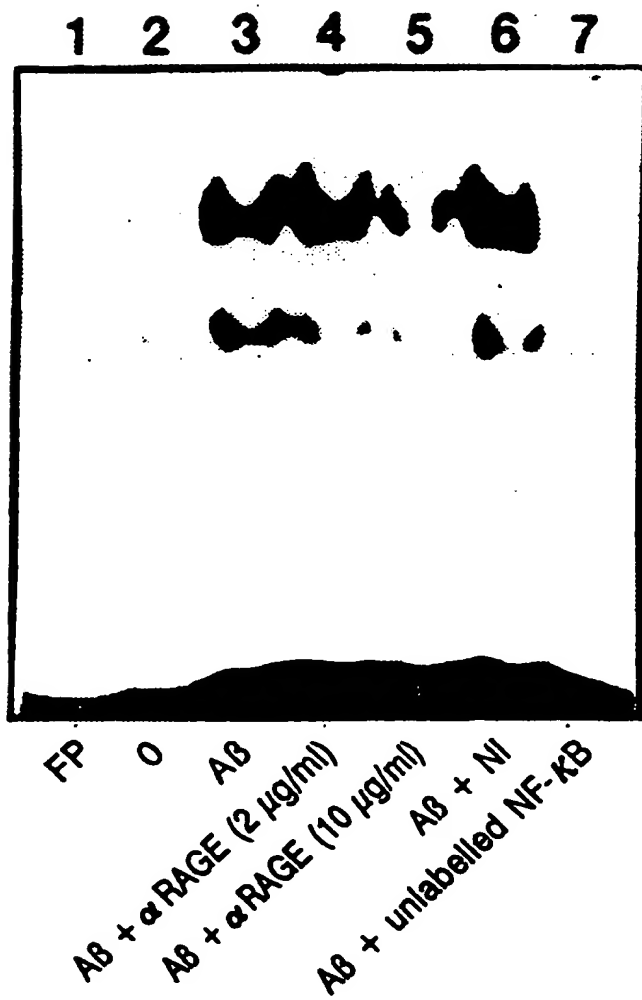
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   424/130.1; 514/2, 44; 530/300, 387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MPSRCH

search terms: advanced glycation end product, receptor, amyloid-beta peptide, inhibit, binding, antibody.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 95/06477 A1, (GLIATECH, INC.), 09 March 1995. See entire document, especially claim 49, page 64.	70-71 ---------- 1-15, 24, 49-68
X -- Y	WO 93/04194 A1, (REGENTS OF THE UNIVERSITY OF MINNESOTA), 04 March 1993. See entire document.	70-71 ---------- 1-15, 24, 49-68
X -- Y	WO 94/17197 A1, (TAKEDA CHEMICAL INDUSTRIES, LTD), 04 August 1994. See entire document.	70-71 ---------- 1-15, 24, 49-68

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 MARCH 1997	1 9 MAY 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MINH-TAM B. DAVIS
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NEEPER et al. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. The Journal of Biological Chemistry. 25 July 1992, Vol. 21, pages 14998-15004. See entire document.	1-15, 24, 49-68, 70-71

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐   Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐   Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐   Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See attached pages.

1. ☐   As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐   As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐   As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒   No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-15, 24, 49-68, 70-71

Remark on Protest
☐   The additional search fees were accompanied by the applicant's protest.
☐   No protest accompanied the payment of additional search fees.

BOX II Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims(s) 1-15, 24, 49-68, and 70-71 drawn to 1) a method for inhibiting interaction of an amyloid-beta peptide with a receptor for advanced glycation end product, comprising contacting the cell with an agent capable of inhibiting said interaction, and 2) a composition comprising said agent.

Group II, claim(s) 16, drawn to a method for inhibiting degeneration of a neuronal cell.

Group III, claim(s) 17-20, drawn to a method for inhibiting formation of an amyloid-beta peptide fibril, or aggregation of amyloid-beta peptide on a cell.

Group IV, claim(s) 21-23, drawn to a method for inhibiting infiltration of a microglial cell into senile plaques, or inhibiting activation of a microglial cell.

Group V, claim(s) 25-48, drawn to a method for treating diseases, comprising administering to the subject an agent capable of inhibiting the interaction of the amyloid-beta peptide with the receptor for advanced glycation end product.

Group VI, claim(s) 69, drawn to a method for inhibiting activation of an NF-kB gene in a cell.

The inventions listed as Groups I-VI do not relate to a single invention concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

In the instant case, the shared inventive concept joining these methods would appear to be inhibiting interaction of an amyloid-beta peptide with a receptor for advanced glycation end product by an agent. In order for the invention of Group I to have unity of invention, it is necessary that the single inventive concept be a contribution over the prior art. However, the teachings of WO 95/06477, WO 93/04194, and WO 94/17197 suggest the use of peptides or antibodies for the inhibition of interaction of an amyloid-beta peptide with a receptor for advanced glycation end product, and thus, defeat unity. In conclusion, the invention of claims 1-71 do not fulfill the requirement for unity of invention, and each method of use forms basis of an individual group.